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General

Phosphodiesterase-4 as a potential drug target

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Phosphodiesterase-4 (PDE4) is the predominant enzyme in some specific cell types that is responsible for the degradation of the second messenger, cAMP. Consequently, PDE4 plays a crucial role in cell signalling and, as such, it has been the target of clinical drug development of various indications, ranging from anti-inflammation to memory enhancement. In this review, the fundamental biological role of PDE4 in intracellular signalling, its tissue distribution and regulation are described. The historical development of various chemical classes of PDE4 inhibitors and the challenges that face these inhibitors as therapeutics are also discussed. Finally, recent advances in the structural biology of PDE4 and their complexes with various inhibitors, as well as its potential impact on the rational design of potent and selective PDE4 inhibitors, are presented.

Keywords: asthma, cardiomyopathy, chronic obstructive pulmonary disease (COPD), cilomilast, crystallography, cyclic AMP (cAMP), drug design, emesis, phosphodiesterase (PDE)4, roflumilast, vasculitis

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1. Phosphodiesterase-4 biology and disease association

1.1 PDE family classification and domain structures

Many biological responses are mediated by the levels of cyclic nucleotides (Figure 1) [1]. These cyclic nucleotides, cyclic adenosine 3′-5′-monophosphate (cAMP) and cyclic guanosine 3′-5′-monophosphate (cGMP), are synthesised by adenylyl cyclases (ACs) and guanylate cyclases (GCs). Typically, adenylyl cyclases are activated by G-protein-coupled receptors (GPCRs) that are coupled to the G_s heterotrimeric GTP-binding protein and ACs are inhibited by GPCRs coupled to G_i. GCs are either soluble or membrane-bound. Soluble GCs are typically activated by the second messenger nitric oxide, and membrane-bound GCs are activated by protein ligands of the natriuretic peptide family [2,3]. In order to further regulate the levels of cAMP and cGMP, all cells have phosphodiesterases (PDEs) that hydrolyse cAMP and cGMP to 5′-AMP and 5′-GMP. In humans, there are 21 different PDE isoforms that are classified into 11 different groups. Fundamentally, these PDEs fall into three categories: those that are specific for cAMP, those specific for cGMP, and those that work on both cAMP and cGMP. In Figure 2, the 11 groups of PDEs are depicted and classified according to their nucleotide selectivity.

As highlighted in Figure 2, PDEs contain multiple domains: each contains a conserved carboxy-terminal domain that catalyses nucleotide hydrolysis and there is great variability among the amino-terminal regulatory domains. The principal domains involved include calmodulin binding domains (PDE1) and cGMP-binding GAF domains (PDE2,5,6,9,10,11). It appears that one key role of GAF domains is to regulate dimerisation [4]. Unique to PDE4 is the upstream conserved regions (UCRs) that is present in all its isoforms. Although the specific functions are still unclear, the UCRs appear to modulate dimerisation and they may bind to signalling molecules such as lipids [5-8].

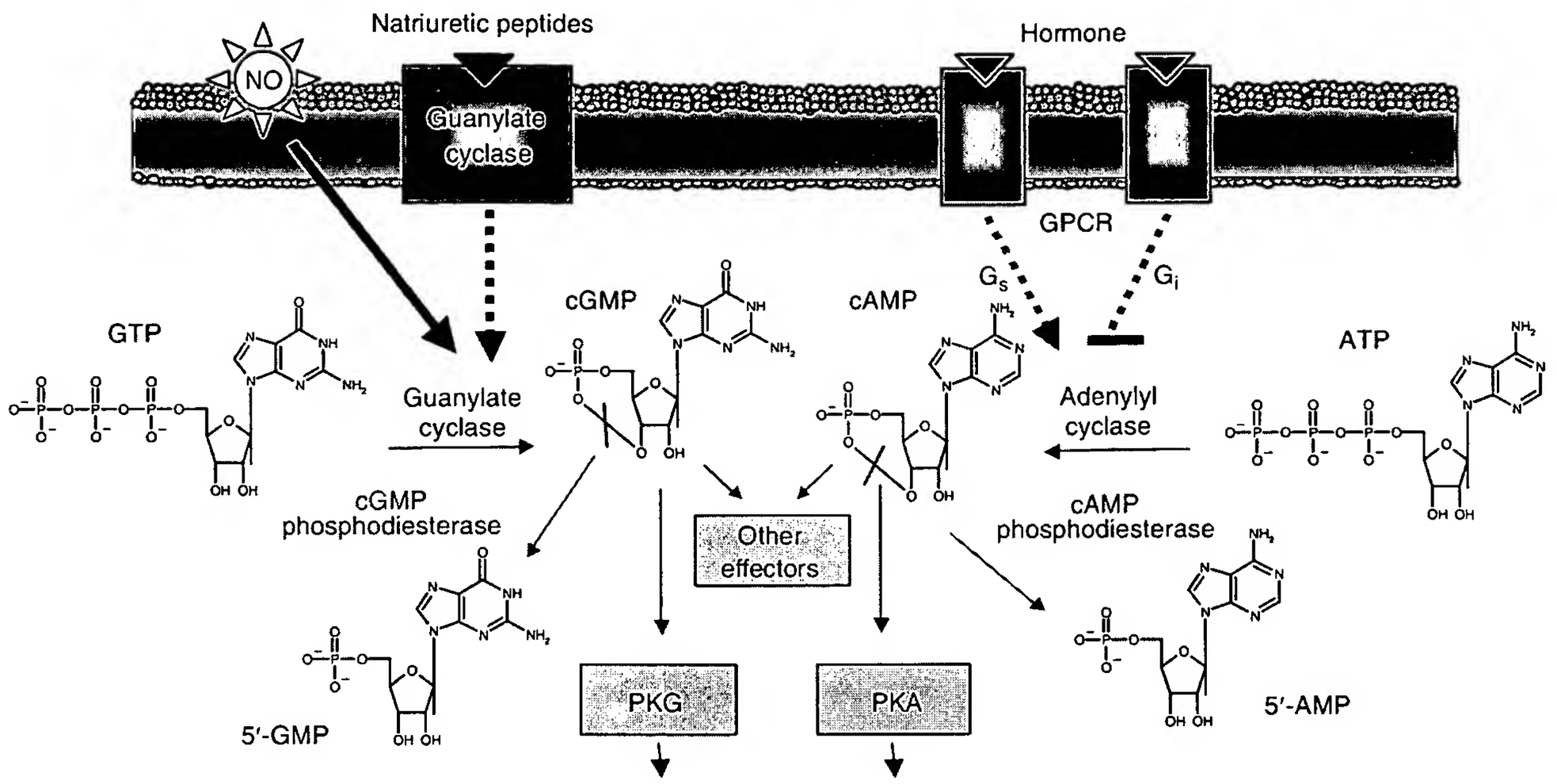

Figure 1. Cyclic nucleotide metabolism. Primary pathways of cyclic nucleotide metabolism are illustrated. Nitric oxide activates soluble guanylate cyclase and activating peptides, such as guanylin or atrial natriuretic peptide, stimulate membrane-bound guanylate cyclase to synthesise cGMP from GTP. A key effector of cGMP is protein kinase G, and the pathway is downregulated by PDE-mediated cGMP hydrolysis to GMP. Hormones (shown in this cartoon by coloured triangles) that activate seven transmembrane G-protein-coupled receptors recruit GTP binding proteins that mediate stimulation or inhibition of adenylyl cyclase. Synthesis of cAMP by adenylyl cyclase results in activation of effectors such as protein kinase A. Again, the pathway is downregulated by PDE-mediated cAMP hydrolysis to AMP. It should be noted that other effector pathways are also stimulated by both cAMP and cGMP.
GPCR: G-protein-coupled receptor; NO: Nitric oxide; PDE: Phosphodiesterase; PKA: Protein kinase A; PKG: Protein kinase G.

1.2 The roles of PDEs in intracellular signalling pathways

As mentioned above, cAMP levels are directly regulated by GPCR modulation of adenylyl cyclase activity. Two primary pathways are known to respond to elevated levels of cAMP: most of the effects have been attributed directly to activation of cAMP-dependent protein kinase (PKA) but recent work also reveals activation of the Rap1 small GTPase via cAMP-responsive exchange factors (EPACs) [9], and activation of cyclic nucleotide-gated ion channels (CNGs) as well. In addition, some studies suggest direct effects of cAMP on ion channels. Among the many GPCRs that lead to elevated cAMP levels are the β-adrenergic and prostaglandin receptors and among the GPCRs that lead to lowering of cAMP levels are some of the muscarinic receptors.

One of the primary effects of PKA activation is the phosphorylation and nuclear translocation of a transcription factor (CREB, cAMP response element binding protein) that binds to cAMP response elements (CREs). In addition, a wide variety of other cellular substrates have been identified, and it is likely that different substrates mediate the various PKA responses in different tissues. Indeed, PDE4 isoforms are themselves substrates for PKA, and this is discussed below.

Although it appears that PKA activation is usually the primary response to elevated cAMP levels, the activation of CNG channels and EPACs may also play important roles. The CNG channels activated by cAMP are pore-forming channels that increase calcium flux and membrane potentials. They are important mediators of the cAMP response in olfactory neurons and sperm cells. Binding of cAMP to EPACs results in activation of the guanine nucleotide exchange activity on Rap1. Rap1 is a key regulator of cellular adhesion in a variety of tissues [10].

1.3 PDE4 isoforms and their tissue distribution and localisation

There are now at least 18 different splice variants of the four PDE4 isoforms (Table 1) [11]. Consequently, cellular and tissue distribution only tell part of the story. Localisation within cellular compartments is equally important in PDE4 biology [12], and in particular gradients of PDE4 activity are constantly in flux, responding to various cellular stimuli including cAMP levels [13]. In addition to the active isoforms, at least one, PDE4A7, lacks catalytic activity and its function remains unclear [14].

PDE4 isozyme distribution has been perhaps most studied in haematopoietic cells, although the expression of PDE4A, PDE4B, and PDE4D isoforms is rather ubiquitous. PDE4C is primarily expressed in testis, skeletal muscle, lung and brain [15]. PDE4A, 4B and 4D are expressed at higher levels in the brain [16,17]. Based on mRNA levels, PDE4B is the predominant

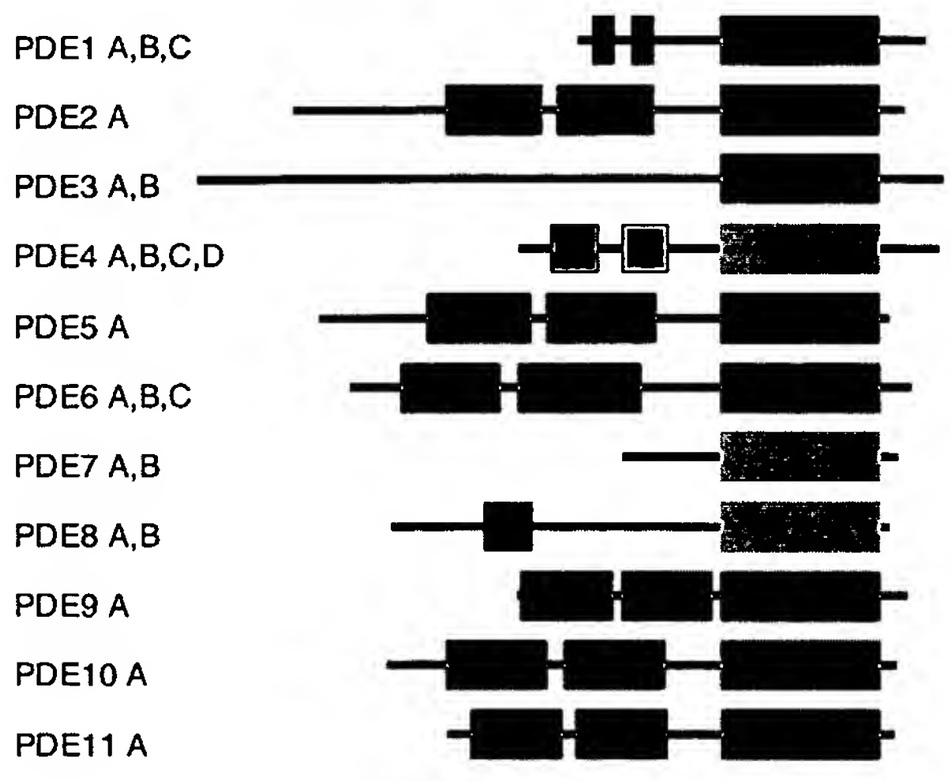

Figure 2. Modular structure of phosphodiesterases. The 11 families of phosphodiesterase are illustrated in cartoon form. The catalytic domain is illustrated in a gradient of colours: the cAMP-selective domains are yellow, the cGMP-selective domains are red, and the dual-selective domains are orange. Upstream regulatory domains are shown in varied colours. The PDE4 upstream regulatory regions 1 and 2 are shown in dark (UCR1) or light (UCR2) grey. GAF domains are shown in green, calmodulin-binding domains are shown in blue, and the 'period, arnt, sim' (PAS) domain is shown in black.
PDE: Phosphodiesterase; UCR: Upstream conserved region.

isoform in neutrophils and monocytes [18]. Expression of the isoforms is tabulated in Table 1.

Subcellular distribution is also an important determinant of PDE function [11]. It has been clearly shown that cAMP can activate PKA in either the membrane or cytosol, depending on where the stimulus occurs [19]. It is one function of PDEs to limit the diffusion of cAMP so that the signal remains localised. In addition to distribution between cytosol and membranes, PDEs have been discretely localised to different regions within cells. This has been nicely illustrated, for example, in olfactory neurons, where PDE4A selectively localises to parts of certain neurons, whereas PDE2A is found in other neurons, and PDE1C is in the cilia [1].

1.4 Modulation of PDE4 catalytic activity by N-terminal domains, phosphorylation and protein binding

As described above, the PDE4 isoforms have N-terminal regulatory domains called UCR1 and UCR2. The most important function of these domains is for regulatory input by PKA [20-22] and extracellular-regulated kinase (ERK) [21,22] as these have been shown to occur in both recombinant and native PDE4 isoforms and to have a functional role. Furthermore, these domains are also important for mediating dimerisation [5,6], and probably for responding to second messengers such as phosphatidic acid [7].

PDE4 isozymes are also regulated by phosphorylation. One important phosphorylation site, namely a site phosphorylated by PKA, lies at the N-terminal end of the UCR1 domain [20-22]. Because this site is directly regulated by cAMP levels, it stands to reason that phosphorylation results in activation of cAMP PDE activity. Indeed, although the effects are modest, PKA phosphorylation does activate PDE4 activity. It should be noted that because the site is within the UCR1 domain, the shorter isoforms of PDE4 do not contain this site for PKA phosphorylation. Nonetheless, all of the long PDE4 isoforms are subject to this form of regulation [23].

A second phosphorylation event occurs at the C-terminal end of the catalytic domain where the mitogen-activated protein kinase ERK2 phosphorylates a serine residue. This event happens in PDE4B, 4C, 4D, but not PDE4A which does not contain the appropriate consensus site. A specific motif in the PDE4 catalytic domain has been identified as a binding site for ERK2 [24]. It appears that phosphorylation of the short forms (e.g., PDE4B2) results in stimulation of activity, whereas activities of long forms (e.g., PDE4B1, PDE4D3) are inhibited [21,22]. In monocytes, long PDE4 activity predominates and activation of cells by pro-inflammatory stimuli causes ERK phosphorylation and inhibition of PDE4D3, which attenuates cell activation by increasing cAMP levels. By contrast, in macrophages PDE4B2 predominates and pro-inflammatory stimuli cause ERK phosphorylation and activation of PDE4B2, which facilitates cell activation by decreasing cAMP levels [25] consistent with PDE4B offering an important therapeutic target.

In addition to intramolecular regulation and post-translational phosphorylation events, PDE4s are also regulated by binding to a set of scaffolding proteins. These include immunophilins such as X-associated protein (XAP)-2 [26], β-arrestin [27], the AKAPs [28] and RACK1 [29]. Specifically, it has been shown that PDE4A5 binds to XAP2 [26] and PDE4D5 binds to RACK1 [29]. Furthermore, β-arrestin-mediated PDE4 cAMP PDE recruitment regulates β-adrenoceptor coupling, switching from G_s to G_i [30]. Each of these binding interactions causes assembly of complexes involving PDE4 isozymes with alternative signalling enzymes. The primary role for PDE4 may be to limit diffusion of cAMP from these signalling complexes [13].

1.5 The roles of PDE4 in the inflammatory response

Because of the critical role of cAMP in mediating cytokine responses, cAMP PDEs are also implicated. As described above, many of the mediators of the inflammatory response, such as T cells, B cells, monocytes, neutrophils, eosinophils and macrophages have PDE4 enzymes as their primary cAMP PDE. Among the inflammatory diseases that are implicated by these cellular mediators are asthma, chronic obstructive pulmonary disease (COPD), rheumatoid arthritis, inflammatory bowel disease, Crohn's disease and multiple sclerosis. Because of the critical involvement of the cAMP

Table 1. Size and tissue distribution of PDE4 isozymes.

Name	Predicted MW (kDa)	Abundant tissue distribution	Accession #
PDE4A1	72	Brain	AAC25679PDE4ARD1
PDE4A4B	98	Inflammatory cells	AAC35012.1
PDE4A7	37	Brain, spleen	AAC35014
PDE4A10	91	Inflammatory cells	AAD34217AF073745_1
PDE4A11	95	Wide expression	AY618547
PDE4B1	82	Lung	CAH70628
PDE4B2	83	Lung, inflammatory cells	NP_002591
PDE4B3	82	Lung	AAB96381
PDE4B4	67	Liver, brain	XP_001862
PDE4C1	80	Lung	NP_000914
PDE4C2	68	Lung	AAD47055
PDE4C3	76	Testis	AAD47054
PDE4D1	66	Spleen, heart	AAA97890
PDE4D2	58	Spleen	AAA97891
PDE4D3	76	Brain, lung, kidney	AAA97892
PDE4D4	91	Brain	NP_006194
PDE4D5	84	Lung, kidney	AAC00069
PDE4D6	59	Brain	AF536975
PDE4D7	84	Brain, testis	AF536976
PDE4D8	77	Lung, heart, liver	AF536977
PDE4D9	77	Spleen, heart, lung	AY245867

MW: Molecular weight; PDE: Phosphodiesterase.

pathway in the inflammatory cells, PDE4 isozymes are likely to play a key role. Consequently, the development of PDE4 inhibitors as therapeutic agents for these diseases has been a major pharmaceutical focus for many years.

The roles of PDE4 in respiratory diseases such as asthma and COPD are at least twofold: PDE4 is an important mediator of the inflammatory cells as described above, and PDE4 also indirectly controls the degree of bronchodilation. In the inflammatory cells, cAMP is a negative regulator of the primary activating pathways, such as cytokine release by T cells. Inhibition of the PDE4 isozymes in these cells results in elevated cAMP levels and consequent inactivation of the inflammatory response. Interestingly, although PDE4 is the predominant cAMP PDE in these cells, the different PDE4 isozymes have different functions as highlighted by the use of targeted disruption of PDE4B and PDE4D in mice. Whereas PDE4D inactivation affects smooth muscle contractility but has little effect on T_H2 cytokines [31], PDE4B ablation blocks T_H2 cytokine and TNF-α production [32]. Although both of these knockout mouse strains have reduced airway hyper-reactivity, clearly the underlying mechanisms are different.

In addition to the direct role of cAMP in inflammatory cell function, elevated cAMP levels also lead to smooth muscle relaxation. Consequently, inhibition of PDE4 activity leading to higher cAMP levels causes bronchodilation, thereby alleviating symptoms of respiratory diseases such as asthma or COPD. It should be noted that PDE3 plays a more direct role in regulating bronchodilation by regulating cyclic nucleotides in airway smooth muscle [33]. Note that PDE4 levels are elevated in response to environmental pollutants such as smoking. For example, PDE4A4 is upregulated in lung macrophages of smokers and both PDE4A4 and PDE4B2 are upregulated in peripheral monocytes from smokers [34]. PDE4B2 levels are significantly elevated in macrophages that have been challenged with the bacterial endotoxin lipopolysaccharide (LPS).

1.6 Memory-enhancing effects of PDE4 inhibition

The connection between PDE4 activity and cognition has been speculated ever since the discovery that the cAMP-regulating *dunce* gene [35] of the fruit fly *Drosophila melanogaster* encodes a PDE4 homologue [36]. The biochemistry of memory is still an active field of research, and perhaps the best-understood aspect involves the *N*-methyl-D-aspartate (NMDA) receptor-mediated pathway. Binding of NMDA to its receptor results in increases of calcium levels that, in turn, through binding to calmodulin, activate AC [37]. The

resulting elevation of cAMP levels causes activation of PKA, phosphorylation of CREB, and ultimately the reorganisation of gene transcription that registers a synaptic plasticity of the affected neuron.

Although it appears that elevated cAMP levels leading to elevated PKA activity are important mediators of synaptic plasticity, the timing of these events is also critical. Thus, activating PKA using a nucleotide analogue mimicking cAMP, does not lead to behavioural memory improvement [38]. By contrast, low level inhibition of PDE activity results in the maintenance of basal levels of cAMP, but an amplification of the transient cAMP spikes at active synapses [39]. Coupled with the earlier findings in the fruit fly [36] that the critical PDE mediating this response is PDE4, the observation that PDE inhibition may correct cognitive impairment has sparked great interest in developing PDE4 inhibitors to address memory disorders [40].

1.7 Cardiovascular and osteogenic roles of PDE4

Two important roles for PDE4D have recently been identified in cardiovascular tissue: a correlation for PDE4 polymorphism with stroke, and an involvement in vascular smooth muscle cell proliferation. In a human genetic analysis of families living in Iceland, a linkage between the PDE4D gene and large vessel occlusive stroke was proposed [41]. This linkage appeared to correlate with increased expression of PDE4D7. Analysis of cohorts from other populations, however, failed to find such a linkage [42,43]. Apparently, further work is needed to clarify the role of PDE4D in stroke.

More recently, it has been shown that PDE4D plays an important role in vascular smooth muscle proliferation [44]. Specifically, the PDE4D1 and PDE4D2 isoforms are dramatically upregulated via histone acetylation during smooth muscle cell differentiation. This result implicates PDE4D as a key regulator of cAMP-mediated inhibition of smooth muscle cell proliferation and migration. From these results, it has been hypothesised that selective inhibition of PDE4D following coronary bypass operations could aid in controlling restenosis that derives from the migration of vascular smooth muscle cells to the site of intervention [44,45].

Finally, recent data establish linkage between PDE4D and osteoporosis [46]. Specifically, single nucleotide polymorphisms mapping to the PDE4D gene are linked to variability in bone mineral density.

2. Development of phosphodiesterase-4 inhibitors

2.1 Historical development of PDE4 inhibitors

For decades nonselective PDE inhibitors like theophylline have been used for the treatment of bronchial asthma. Side effects of theophylline include cardiac dysarhythmias and nausea which are believed to be the result of nonselectivity among PDEs, as well as concomitant adenosine receptor antagonism [47-49]. Of the 11 known isozymes of the PDE family, PDE4 appears to be the predominant in various inflammatory cells and, hence, the genesis of selective PDE4 inhibitors.

First-generation PDE4 inhibitors include rolipram (1) and Ro20-1724 (2) that belong to the catechol diether class (Figure 3). The observed side effects of these earlier compounds, including nausea, vomiting and increased gastric acid secretion [50,51] halted their development as therapeutics for the treatment of COPD. As a result, the contemporary drug discovery efforts in PDE4 have involved the design of novel inhibitors with reduced side effects while maintaining the anti-inflammatory properties of rolipram.

Cilomilast (3), roflumilast (4), lirimilast (5) and AWD-12-281 (6), among others, belong to second-generation PDE4 inhibitors (Figure 3). Although these second-generation inhibitors have demonstrated improved side effect profile in animal models and advanced to the level of testing in humans, many of them have been discontinued in the clinic due to narrow therapeutic index. However, roflumilast has reached the stage of preregistration for the treatment of asthma and COPD, and cilomilast has been filed for the approval as a drug for COPD, highlighting the progress in the development of PDE4 inhibitors as therapeutics.

PDE4 exists as four genetically distinct subtypes (PDE4A-D) [11] and this offers a potential for third generation inhibitors, (i.e., subtype-selective inhibitors). It has been shown in the literature that some compounds can target specific inflammatory cell functions [52]. This hypothesis has yet to be proven in clinic.

2.2 Chemical classes of PDE4 inhibitors

PDE4 inhibitors can be divided into distinct classes based on structural motifs present in the molecules (e.g., xanthine, nitraquazone, catechol diethers etc). Generally nonselective for PDE4, xanthine derivatives have been known for some time (Figure 4). These compounds have clear structural similarities to the natural substrate for PDE4, cAMP. Although caffeine (7), theophyline (8) and theobromine (9) represent the natural product class of xanthines with PDE inhibitory potency, denbufylline (10), 3-isobutyl-1-methylxanthine (IBMX, 11) and arofylline (12) are examples of compounds discovered through medicinal chemistry and developed specifically as PDE inhibitors [201]. Nitraquazone was developed by Syntex as a PDE4 inhibitor for the treatment of inflammation in the mid 1980s [53], maintaining some structural similarities to the xanthine-derived PDE4 inhibitors. Nitraquazone represents a divergent class of PDE4 inhibitors, including nitraquazone (13), RS-17597 (14), CP-77059 (15), KF-17625 (16), and compound 17 developed by Almirall, many of which are more selective inhibitors for PDE4 than their xanthine cousins. Compound 17 belongs to a 2,5-dihydropyrazolo[4,3-c]quinolin-3-ones series and exhibited better selectivity versus PDE3 and low emetic potential [54].

Arguably, the most widely represented class of inhibitors belongs to the rolipram family of catechol diethers (Figure 5). rolipram selectively inhibits PDE4 (K_i = 1 μM) displaying

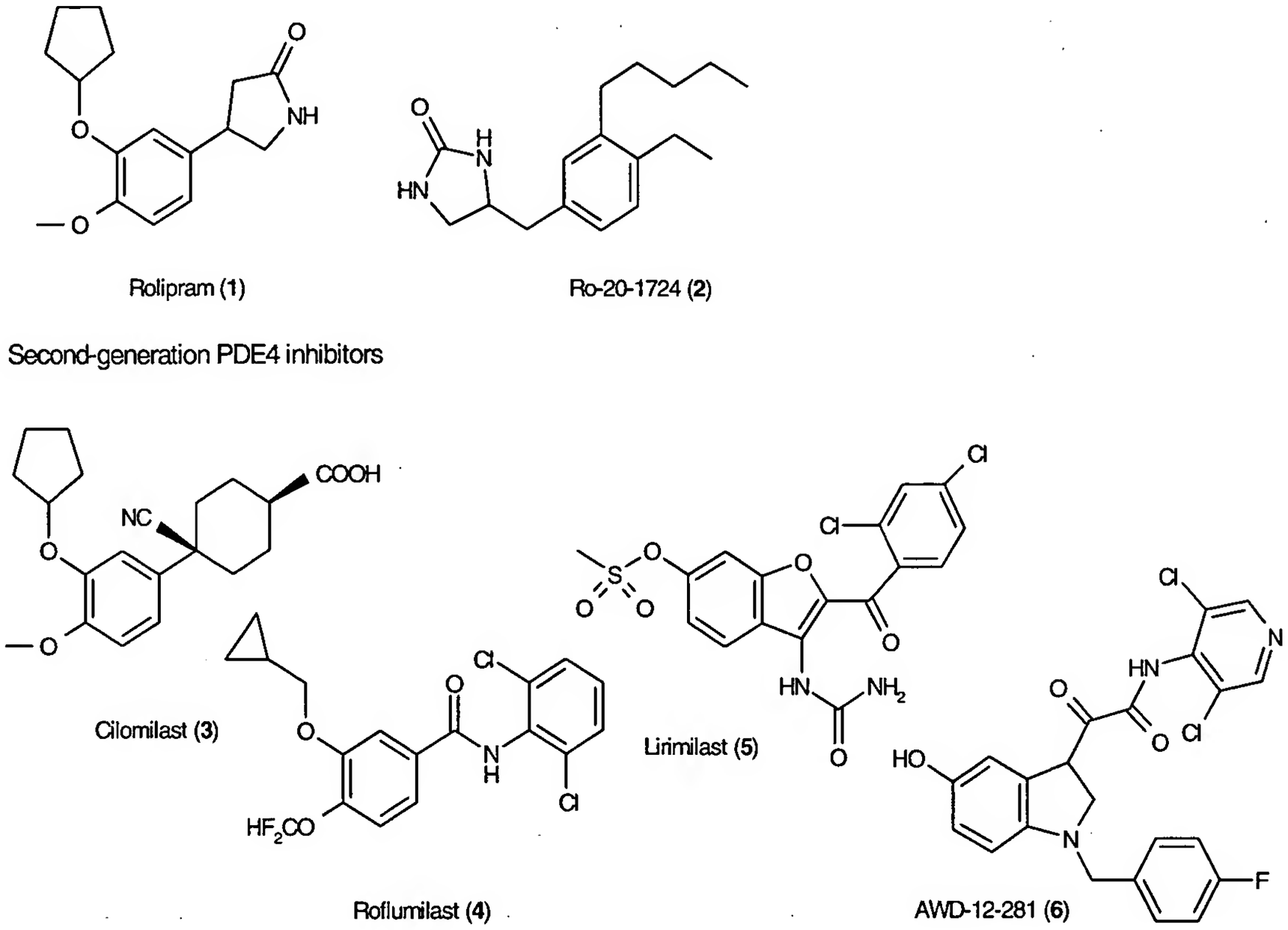

Figure 3. Phosphodiesterase-4 inhibitor development. Selective examples of the first- and second-generation PDE4 inhibitors are shown.
PDE4: Phosphodiesterase-4.

100-fold selectivity over other PDE enzymes [55]. Many examples of catechol diether-containing compounds are reported as PDE4 inhibitors. Some of the better known examples are represented in Figure 5. Mesopram (daxalipram, 18) was discovered as an improved rolipram analogue by Schering as a potential treatment for MS [202]. However, Schering has discontinued further development of Mesopram. Zardaverine (19) was developed by Byk-Gulden as a dual inhibitor of PDE3 and PDE4 [56]. Piclamilast (20) was developed by Rhône-Poulenc as a treatment for arthritis [203]. Piclamilast is a very potent inhibitor of PDE4 (0.7 nM) [57]; however, development of this compound has been discontinued due to toxic side effects. Cilomilast (3) and roflumilast (4), as described in Section 2.1 of this review, are currently in development for COPD. Several groups have designed compounds that are extended versions of the catechol series, such as the rolipram derivative described by Keller (21) [58], Celltech's CT-5357 (22) and pumafentrine (23), a dual PDE4/PDE3 inhibitor from Altana. Filaminast was developed by Wyeth for the treatment of asthma and it has been discontinued after Phase II trials [204]. Otsuka is developing Tetomilast (OPC-6535, 26) as a once daily oral formulation for potential treatment of ulcerative colitis (UC) and COPD and currently in Phase III clinical trials for UC [59]. IC-485 (27) and CDP-840 (28) also belong to the catechol diether family of PDE4 inhibitors. The clinical development of these compounds was stopped due to lack of efficacy or side effects. IC-485 failed to meet the primary end point of improving lung function in Phase II COPD trials and the development was discontinued in March 2005. Orally-active and highly selective CDP-840 was developed by Celltech and Merck Frosst for asthma. Although CDP-840 showed statistically significant inhibition of late-phase allergic response, both companies felt that the treatment effects did not represent a significant therapeutic advantage and further development of this compound was abandoned in 2001.

Several compounds have been developed as PDE4 inhibitors that do not fall into any one particular class of compounds described above (Figure 6). Bayer has developed lirimilast (BAY-19-8004, 5), a novel sulfinate-containing compound for treatment of COPD. ICOS compound (29) represents a novel class of pyrazole and fused pyrazole inhibitors [205,206]. YM-976 (30) from Yamanouchi is a pyridopyrimidinone derivative, which is reported to be non-emetic and was in Phase I clinical trials in 1998 and discontinued in 1999. Tofimilast (31) from Pfizer is an indazole derivative

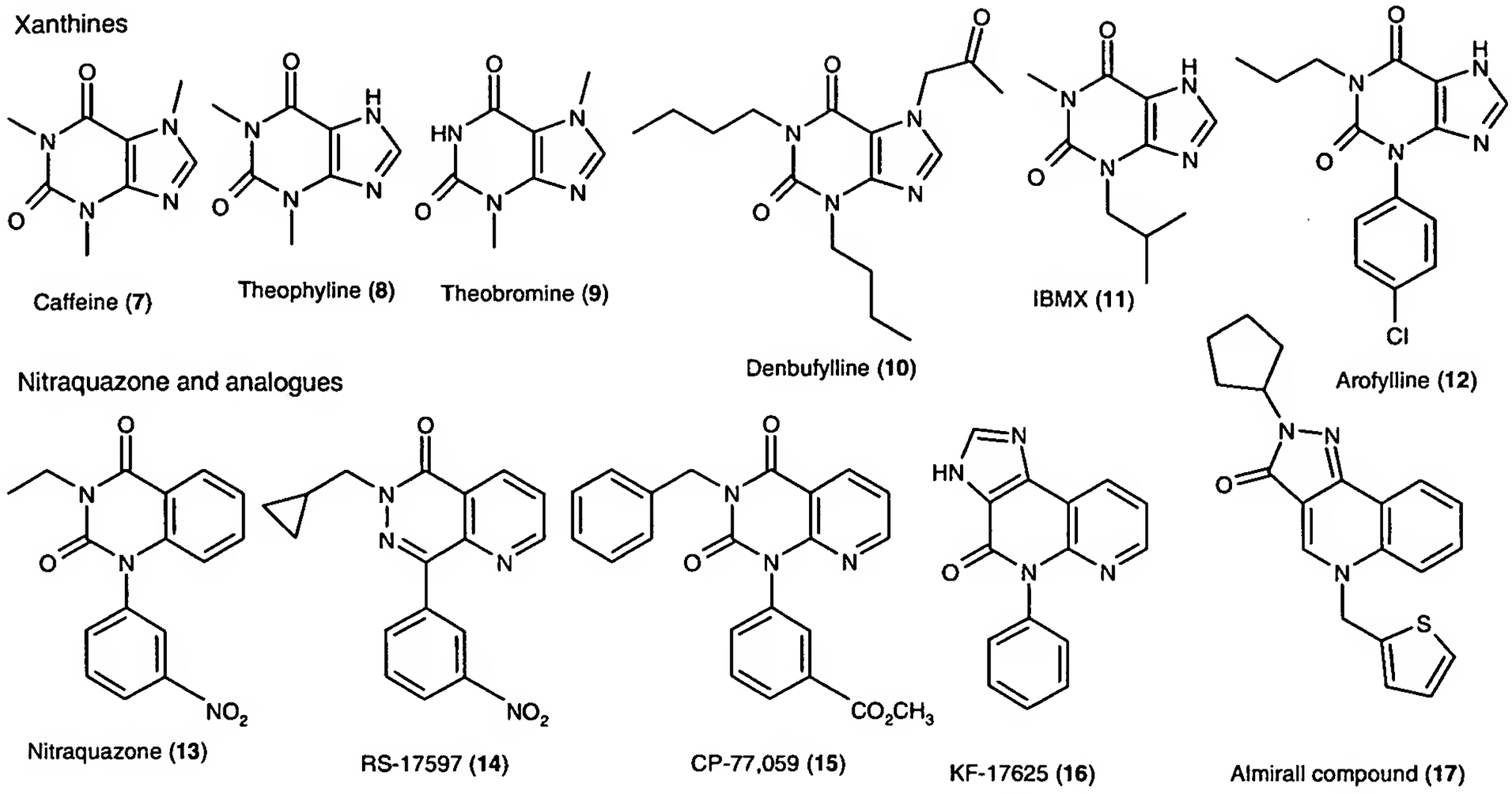

Figure 4. Xanthine derivatives and nitraquzone analogues as phosphodiesterase-4 inhibitors.

that is currently under clinical development. Ibudilast (32), a pyrazolopyridine compound and a marketed drug as a vasodilator and for the treatment of allergic ophthalmic disease in Japan, is currently in Phase II clinical trials for multiple sclerosis by MediciNova under liscence from Kyorin [207]. BAY-61-9987 belongs to imidazotriazinones class of compounds and is a low-affinity rolipram binding site (LARBS) selective inhibitor that is under development by Bayer for the potential treatment of respiratory diseases [208]. These compounds represent new chemotypes that can act as potent and selective PDE4 inhibitors.

Although NVP-ABE171 (33) and CP-671305 (34) are only in the discovery phase and both compounds are selective inhibitors of the PDE4D subtype. NVP-ABE171 is shown to be > 20-times more selective for PDE4D over PDE4B and inhibited the activation and influx of airway neutrophil with an ED_{50} in the range of 3 mg/kg [60]. CP-671305 is also a PDE4D selective inhibitor with a selectivity of more than 95X over PDE4B with a moderate-to-high oral bioavailability in rat, dogs and monkeys [61].

2.3 PDE4 inhibitors for asthma and COPD

Asthma is a chronic lung disease with inflamed and hypersensitive airways that cannot be cured, but controlled. COPD (also known as chronic obstructive airways disease; chronic obstructive lung disease; chronic airflow limitation and chronic airflow obstruction) is a disorder that is characterised by reduced maximal expiratory flow and slow forced emptying of the lungs. PDE4 inhibitors have been shown to relax airway smooth muscle and to suppress the activation of inflammatory cells [62,63]. PDE4 inhibitors that are at various stages of clinical development are cilomilast, roflumilast, AWD-12-281 (6), CC-10004, ONO-6126 (24) and GRC-3886.

The two PDE4 inhibitors at the most advanced stage of development are cilomilast and roflumilast (BY-217, APTA-2217), for the treatment of COPD and asthma/COPD, respectively.

GlaxoSmithKline (GSK) submitted an NDA in December 2002 for Cilomilast and received an approvable letter from FDA. Following the letter, the FDA requested that GSK conduct long-term efficacy studies of 6 months or 1 year. Cilomilast is a potent (IC_{50} = 95 nM) orally bioavailable PDE4 inhibitor with a half-life of 7 h in humans. In a Phase III study, cilomilast showed (15 mg p.o. b.i.d. for 6 months, 2058 stable COPD patients) a sustained improvement in lung function and a 40% reduction in the risk of exacerbations [64,65]. Four pivotal studies of cilomilast were performed by GSK (2263 patients), of which two of the studies had reached clinical significance, with a mean change of the forced expiratory volume over 1 minute (FEV1) of 10 ml from baseline in both trials of cilomilast, compared with a 30 ml reduction for placebo.

A European filing was submitted for roflumilast in February 2004 for COPD and the US NDA is expected to be filed in 2006. Roflumilast is a highly potent (IC_{50} = 0.8 nM) orally bioavailable (F = 79% in human) PDE4 inhibitor. An *in vivo* metabolite of roflumilast, the N-oxide, is equally active and has a longer terminal half-life. In the last two years results of various clinical trials of roflumilast for asthma and COPD have appeared in abstract presentations. In a double-blind, 3-period crossover study, 28 patients

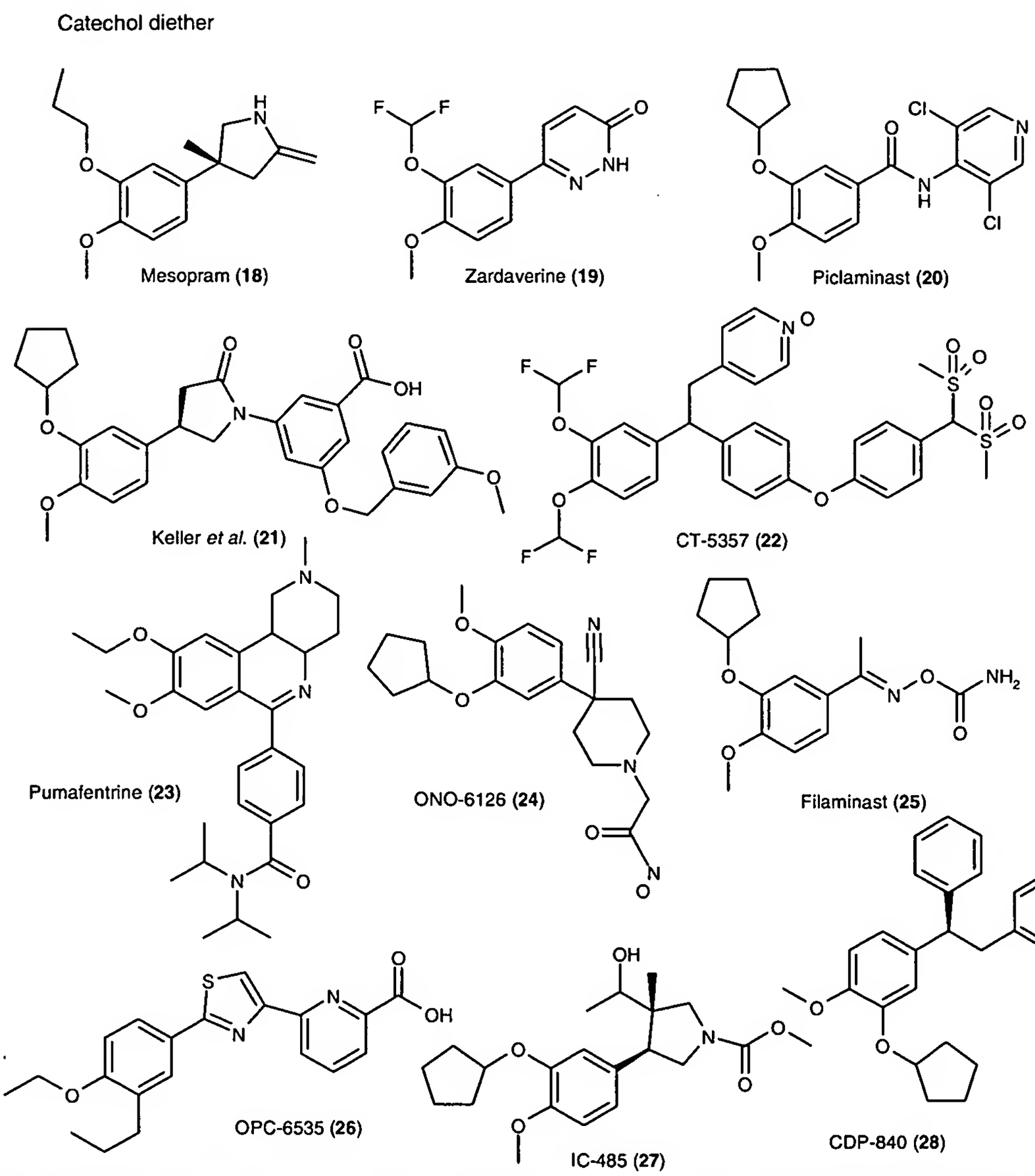

Figure 5. Catechol diethers as phosphodiesterase-4 inhibitors.

received 250 or 500 µg/day of roflumilast, or placebo. Roflumilast was well-tolerated and showed dose-dependent reduction in the late asthmatic response compared with placebo with no changes in vital signs and most adverse events were mild-to-moderate. In another study, roflumilast (500 µg, 456 patients) showed constant efficacy, as assessed by FEV1 measurement, over one year with no increased incidence of vomiting. In September 2003, Altana has completed a Phase III study (RECORD) with roflumilast in 1400 patients with COPD (250 and 500 µg, 24 weeks) which has improved lung functions and was well-tolerated [64,66,67]. Results from the first one-year study 'RATIO/M2-112' in 1513 patients with very severe COPD was announced by Altana on July 2005. Although the primary end point of lung function (FEV1) was significantly improved with roflumilast treatment compared to placebo the co-primary end point, the frequency of moderate and severe exacerbations, had no statistically significant reduction in the rate of total exacerbations. The most common adverse events observed in this study associated with roflumilast treatment were nausea, headache and diarrhoea. Consequently, Pfizer have withdrawn from their collaboration with Altana on the co-development of roflumilast because of lack of efficacy on COPD excacerbations.

AWD-12-281 (GW-842470), a 5-hydroxyindole derivative, is a selective inhibitor of PDE4 (IC_{50} = 7 nM) developed by Elbion and GSK for the treatment of COPD with an inhaled formulation. AWD-12-281 inhibited LPS-induced

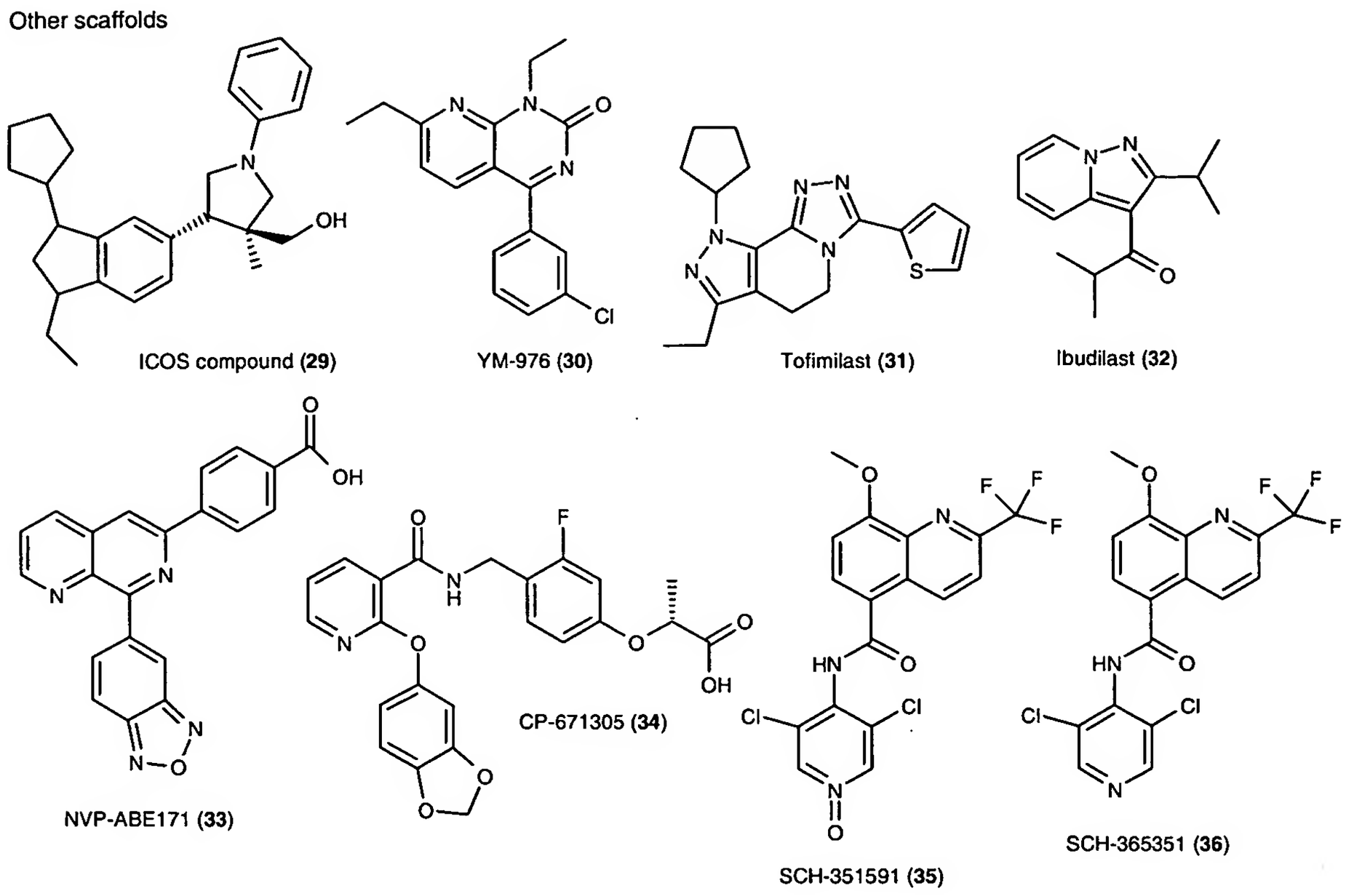

Figure 6. Other classes of phosphodiesterase-4 inhibitors.

and allergen-induced TNF-α release with IC_{50} values of 100 nM and 90 nM, respectively. Intratracheal administration of 30 mg/kg dose of AWD-12-281 inhibited allergen-induced eosinophil infiltration in rats. Intratracheal doses up to 10 mg/kg in ferrets and dogs had no evidence of emesis. In Phase I clinical trial adverse events in patients dosed up to 40 mg/kg/day were similar to those in placebo group. AWD-12-281 was safe and well-tolerated in humans [68].

CC-10004, a thalidomide analogue currently in Phase II clinical trials for asthma and COPD (PDE4 IC_{50} = 74 nM), is developed by Celgene [69-71]. It was safe and well-tolerated in a Phase I safety and pharmacokinetic study. When dosed at 20 mg, circulating levels of CC-10004 was higher than the *in vitro* human whole blood IC_{50} value for 14 h indicating the potential for once- or twice-daily dosing. At a dose of 25 mg/kg once a day oral dosing CC-10004 reduced hind paw thickness comparable to dexamethasone dosed at 1 mg/kg in a collagen antibody-induced murine arthritis model.

ONO-6126 (24), a piperidine hydroxamate compound with structural similarity to Cilomilast is under development by Ono Pharmaceutical for the potential treatment of asthma and COPD (PDE4 IC_{50} = 0.080 +/- 0.050 nM). ONO-6126 inhibited LPS-induced TNF-α release in human, dog and guinea-pig peripheral blood (IC_{50} = 330 nM – 770 nM) in a preclinical evaluation. It had significant activity on oral dosing at 0.1 mg/kg with emesis appearing at 1.0 mg/kg. Activity was also demonstrated in a guinea-pig asthma model (0.3 mg/kg once-daily for 2 weeks) [72]. ONO-6126 demonstrated increasing suppressive effects on TNF-α release upon repeated oral administration (3 or 9 mg b.i.d. for 7 days) to healthy male subjects based on a report from a Phase I clinical study [73].

GRC-3886 is developed by Glenmark, Forest and Teijin for the potential treatment of asthma and COPD. It inhibits the PDE4 isozyme subtypes A, B, C and D with IC_{50} values of 1.36, 2.56, 6.17 and 1.7 nM, respectively [74]. GRC-3886 has a good oral bioavailability across various animal species and no emetic effect had been noted at oral doses of up to 100 mg/kg. In September 2004, it was reported that GRC-3886 had entered Phase I trials and the studies were completed in January 2005. The drug was safe, well-tolerated and had excellent pharmacokinetics, with a long half-life, suggesting a once-daily dosing regimen would be suitable for future studies.

2.4 PDE4 inhibitors for memory enhancement

As described in Section 1.6, PDE4 plays an important role in certain aspects of cognition. Indeed, in animal models of memory, rolipram has been shown to rescue the cognitive capacity of rodents that have been challenged with the muscarinic receptor antagonist scopolamine [75]. Consequently, a

number of programmes aimed at developing PDE4 inhibitors to pharmacologically enhance cognition have been initiated and studies with newer PDE4 inhibitors also show cognitive enhancement in animal models [40,76]. MEM1414 was selected by Roche from Memory Pharmaceuticals as clinical candidate and Phase I trial had been initiated by September, 2003. Memory has plans to evaluate the data to determine the future of this drug. Development of PDE4 inhibitors in memory enhancement have also been described by Helicon Pharmaceuticals, HT-0712 (IPL-455903) licensed from Inflazyme [40]. Phase I trial began in December, 2004, to assess the safety, tolerability and pharmacokinetic properties of escalating single oral doses of the drug in 50 healthy volunteers. Results released in May 2005 showed that the drug was safe, well-tolerated and did not cause nausea. The effect of food on the absorption of the drug was found to be minimal, and it had showed acceptable pharmacokinetic properties. Currently, Helicon plan to continue the Phase I programme with an additional double-blind, placebo-controlled, multi-dose, two-week trial in healthy volunteers.

2.5 Challenges facing PDE4 inhibitor development

2.5.1 Emesis as a dose-limiting side effect of PDE4 inhibitors

Ever since rolipram was developed clinically as an antidepressant over two decades ago, the side effects of nausea and emesis have plagued the PDE4 inhibitor field. For this reason, preclinical studies to determine the emetic potential of PDE4 inhibitors are extensively reported. Historically, the ferret is a preferred species for evaluating emesis, and therapeutic index can be determined by comparing with effects on pulmonary mechanics in the ferret. To better understand the mechanisms leading to emesis, a particularly emetic PDE4 inhibitor was developed as a photoaffinity probe [77]. Further mechanistic studies identified the noradrenergic pathway as a mediator of the emetic response, and enabled the development of a mouse model that correlates well with the ferret response [78].

Although still controversial, there is increasing evidence that PDE4D may be responsible for some of the more serious side effects of PDE4 inhibition. It has been recognised for some time now that an array of PDE4 isoforms is expressed in inflammatory cells [79]. Knockout studies have shown that whereas PDE4D inactivation affects smooth muscle contractility, but has little effect on T_H2 cytokines [31], PDE4B ablation blocks T_H2 cytokine and TNF-α production [32]. From the viewpoint of CNS side effects, PDE4B does not affect α2-adrenoreceptor-mediated anaesthesia, a surrogate of emesis in rodents [80]. Conversely, ablation of PDE4D impacts this response, suggesting that PDE4D, but not PDE4B, inhibition, may be responsible for the emetic effects of nonselective PDE4 inhibitors.

Another strategy is based upon the observation that PDE4 enzymes exist in both low- and high-affinity rolipram-binding conformations [81]. Inhibition of the high-affinity rolipram binding site (HARBS) appears to correlate with the side effects [82]. Compounds with selectivity for the low-affinity form of the enzyme (LARBS) are expected to have better therapeutic index compared to rolipram [83,84]. In cellular assays, the HARBS conformation is stabilised through binding to the tyrosine kinase Lyn [85]. Although the precise structural determination of the HARBS conformation has not been elucidated, a role for magnesium ion binding in regulating this conformation has been suggested [86]. Scientists from SmithKline Beecham evaluated the selectivity in binding to LARBS and HARBS for a number of compounds using eight different assays spread among five species to identify compounds for treating inflammation or for dilating bronchi [209].

Although the knockout studies suggest that a selective PDE4B inhibitor would be beneficial to treat inflammatory diseases, very limited information is available about discovering a subtype-selective inhibitor. However, a group from SKB published their finding on the correlation between inhibition of PDE4A and PDE4B and suppression of inflammatory cell function [52]. The functional role of PDE4A, PDE4B and PDE4D were explored using ten subtype-selective inhibitors that are grouped into two categories: (a) dual PDE4A/PDE4B inhibitors and (b) PDE4D inhibitors. All these compounds were evaluated for their ability to inhibit antigen-stimulated T cell proliferation and LPS-induced TNF-α release from peripheral blood monocytes and the results were correlated with their ability to inhibit the catalytic activity of recombinant human PDE4A, PDE4B and PDE4D enzymes. The results suggest that PDE4A and/or PDE4B may play a major role in regulating the two inflammatory cell functions. As discussed earlier, the abundance of PDE4B2 and its activation by ERK phosphorylation in macrophages [87] gives a fundamental insight into why PDE4B may be a special target. More recently, selective targeting of PDE4 isoforms by siRNA has provided further evidence that PDE4B and PDE4D can have differential roles within the same cell [88].

2.5.2 Cardiac abnormalities linked to chronic PDE4D inhibition

A selective role for PDE4D has been implicated in heart tissues. Thus, it has been shown that cAMP levels controlled by PDE4D are directly linked to β2-adrenergic receptor signalling in cardiac myocytes: PDE4D-regulated activation of PKA selectively mediates β2- and not β1-adrenoreceptor signalling [89]. These results implicate PDE4D as an important regulator of cardiac myocyte contraction rate.

More recently, long-term inhibition of PDE4D has been linked to severe cardiac defects, calling into question the chronic systemic use of nonselective PDE4 inhibitors [90]. Specifically, it has been previously shown that PKA-dependent hyperphosphorylation of the ryanodine receptor leads to cardiac arrhythmias. PDE4D activity is selectively decreased in patients suffering from heart failure. In ageing PDE4D knockout mice, the animals develop progressive cardiomyopathy, accelerated heart failure following myocardial infarction

and cardiac arrhythmias. Previous experience with promiscuous PDE inhibitors, such as theophylline, or PDE3-selective inhibitors have shown increased cardiac symptoms, and it remains to be seen if these rather troubling symptoms will be seen in human patients on PDE4-selective therapy.

2.5.3 Vasculitis as a dose-limiting side effect of PDE4 inhibitors

Vasculitis is a general term for a group of uncommon diseases which feature inflammation of the blood vessels and is characterised by inflammation in and damage to the walls of various blood vessels. The actual cause of these vasculitis diseases is not known and each form of vasculitis has its own characteristic pattern of symptoms.

Clinical investigation of PDE inhibitors has been hindered by their association with an insidious vascular toxicity in nonclinical animal models. For example, very high doses PDE4 inhibitors have been found to induce lesions in splanchnic arteries in rats [91]. Histopathological characterisation of mesenteric vasculitis induced by PDE inhibitors is by medial necrosis and haemorrhage with perivascular oedema mixed inflammatory cell infiltration. The pathogenesis of vasculitis induced by PDE inhibitors is currently poorly understood. Apoptosis has not been extensively evaluated in rats given PDE4 inhibitors, however, and mediators of apoptotic signalling have not been fully described.

Toxicity of repeat dose of rolipram in rats was studies by a group at RPR [91]. In this study, rolipram was administered orally to female rats at 0, 10, 30 or 100 mg/kg for 14 days. One treatment-related death in the 100 mg/kg dose group was observed on day 3 and all rats at this dose level were euthanised on day 5. Several clinical signs were observed in treated rats including increased salivation, slight distension of the abdomen, emaciated appearance, and ataxia. The results of this study demonstrate that rolipram can cause effects on the heart and vasculature of rats.

CI-1018 is another PDE4 inhibitor that was evaluated for vasculitis side effects in rats [92]. In this study, vasculitis was evaluated using a metabonomic approach which is an emerging technology with great potential for rapid noninvasive assessment of toxicity *in vivo* and for providing identification of peripheral surrogate markers of toxicity. In this study, CI-1018 was administered for up to 4 days to groups of male Wistar rats at doses up to 3000 mg/kg and urine was collected from all animals pretest and daily for metabonomic analysis. CI-1018 was found to be not a particulaly potent vasculitis-inducing agent, although the vascular changes produced by the compound were similar to those reported for rolipram. Recently, two publications from Pfizer provided new insights into the mechanism of vasculitis [93] and involvement of nitrative stress in mesenteric vasculitis [94] in rats.

SCH-351591 (35), a selective potent inhibitor (IC_{50} = 58 nM) of PDE4 under investigation as a potential therapeutic for asthma and COPD, caused arteriopathy in both rats [95] and monkeys [96]. In the vehicle-controlled, three-month, dose-escalated monkey study, SCH-351591 was dosed at 12, 24, or 48 mg/kg (4 monkeys/group) daily. Acute to chronic inflammation of small-to-medium sized arteries in various organs and tissues were observed in this study. Early mortality observed in low and mid dose group in this study was attributed to sepsis or colon inflammation. The major metabolite of SCH-351591 is the des-oxy analogue (36, SCH-365351) which is also a very potent inhibitor (IC_{50} = 20 nM) of PDE4. The plasma concentrations for both parent and metabolite achieved in this study were within or less than the 10 – 20 uM range. The authors suggested that the possibility of nonspecific PDE inhibition may have contributed to the toxicity observed in this study. Further development of SCH-351591 was halted by Schering-Plough in October, 2002.

3. Structural basis for the rational design of phosphodiesterase-4 inhibitors

3.1 PDE4 catalytic domain structures

The knowledge of the 3D structure of PDE4 is crucial for the rational design of PDE4 inhibitors. Although the structure of the full-length PDE4 would be more desirable for such a purpose, it has eluded us so far due to the difficulty of obtaining sufficient amounts of soluble full-length PDE4 for structural studies. Tryptic digest analysis was used to identify the compact core catalytic domain of the short isoform PDE4B2B and this has led to the first crystal structure of the PDE family, the apo catalytic domain of PDE4B [97]. This crystal structure has revealed that the catalytic domain of PDE4B adopts a compact α-helical structure consisting of 16 helices that can be divided into 3 subdomains (Figure 7a). The active site forms a deep pocket located at the junction of the three subdomains and is lined with highly conserved residues (Figure 7b). There are two metal ions coordinated by residues from all three subdomains and these interactions seem to hold the three domains together. The first metal ion is a zinc ion (Zn^{2+}) which is coordinated by H238, H274, D275, D392 and one water molecule. These two histidines and two aspartates are absolutely conserved across all the PDE family members. The second metal ion is most probably a magnesium ion (Mg^{2+}) which is coordinated by the same D275 that also coordinates to Zn^{2+}, and four water molecules, one of which bridges the Mg^{2+} and Zn^{2+}. Although the Zn^{2+} and Mg^{2+} are found to be five-coordinated in a trigonal bipyramid configuration in the first apo PDE4B structure, subsequent structures of the catalytic domain of many other PDEs including PDE4B have revealed a near ideal octahedral coordination geometry for both Zn^{2+} and Mg^{2+}. The absence of two water molecules in the coordination sphere of the two metal ions in the apo structure of PDE4B was probably caused by the binding of two arsenate ions from the crystallisation buffer in the active site. It was suggested that the position of the arsenate near the di-metal ions represent a possible binding site for phosphate. This was later confirmed by several co-crystal structures of PDE4B in complex with

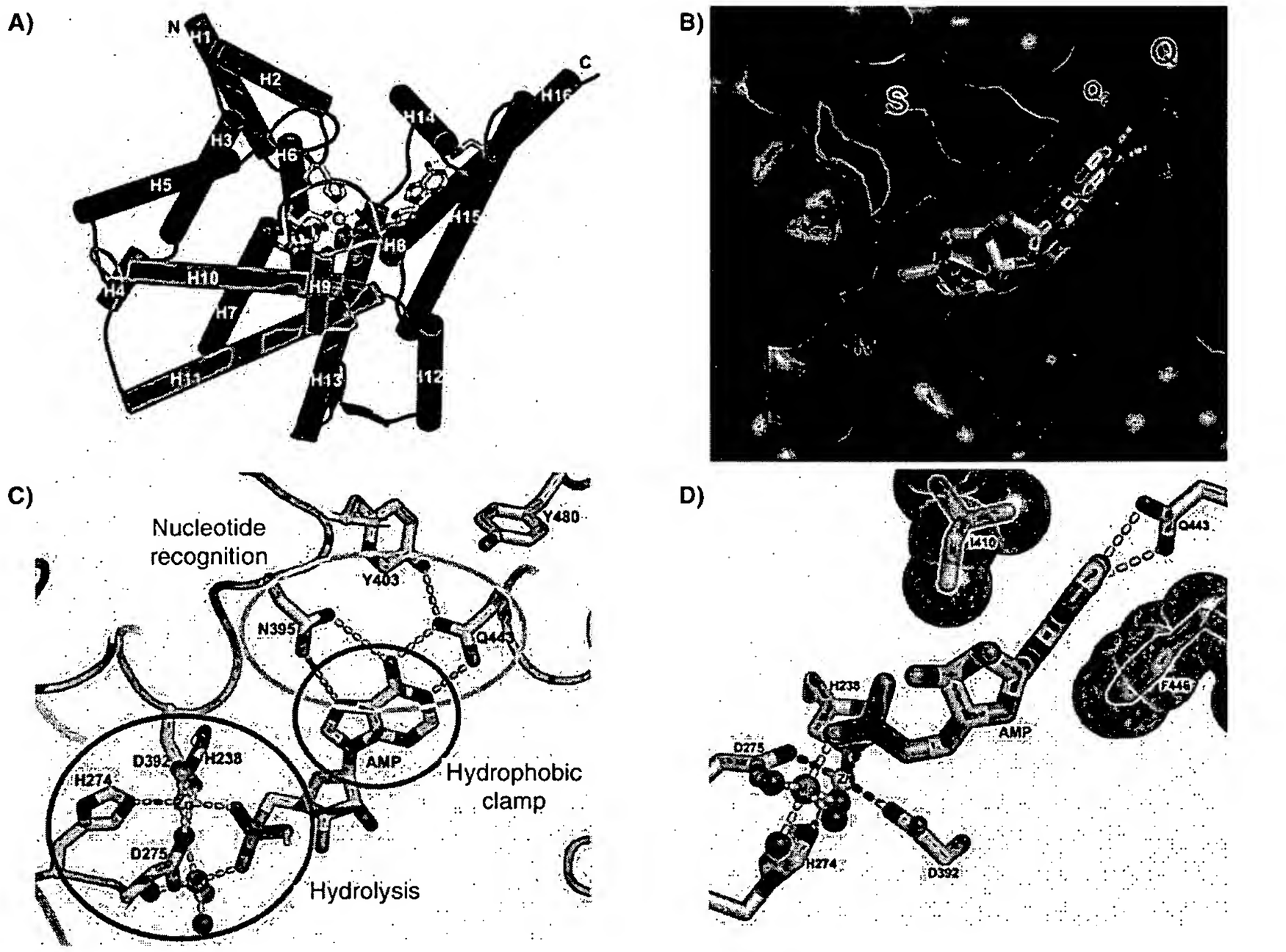

Figure 7. PDE4 structure, substrate and inhibitor binding to the active site. A) A cartoon representation of the overall structure of the catalytic domain of PDE4B. The α-helices are represented by cylinders and the loops are represented by tubes. The α-helices labelled 1 – 16 are divided into three subdomains coloured blue, green and red, respectively. The Zn^{2+} and Mg^{2+} are represented by yellow and magenta spheres. Residues coordinating the metal ions, as well as bound AMP, are shown in stick models. **B**) A surface rendition of the PDE4B active site is shown with bound AMP in stick model. The active is divided into three subpockets: Q, S and M and coloured in red, green and blue, respectively. The Q-subpocket can be further divided into Q1, Q2 and QP regions. The yellow and magenta surfaces are that of Zn^{2+} and Mg^{2+} ions. **C**) Residues in the active site can be clustered into three functional groups that are responsible for: (1) nucleotide recognition; (2) hydrophobic clamp; (3) hydrolysis. These are highlighted on the co-crystal structure of PDE4B bound to AMP. **D**) The purine ring of AMP is sandwiched by the hydrophobic clamp formed by two highly conserved residues, I410 and F446 in PDE4B. Residues I410 and F446 are represented by sticks with semitransparent sphere models superimposed.
AMP: Adenosine monophosphate; PDE: Phosphodiesterase.
Adapted from HOUSLAY MD, SCHAFER P, ZHANG KYJ: Phosphodiesterase-4 as a therapeutic target. *Drug Discov. Today* (2005) **10**:1503-1519 [114], with permission from Elsevier.

AMP [98-100]. The crystal structure has revealed that the residues coordinating both Zn^{2+} and Mg^{2+} ions are from two highly conserved PDE sequence motifs HNXXH and HDXXH, each of which was previously thought to form a separate metal binding site [101]. Through a computational docking of cAMP into the active site of PDE4B, Xu *et al.* have predicted the binding mode of cAMP, as well as residues involved in hydrolysis and nucleotide recognition. All of these predictions were borne out later through the co-crystal structures of enzyme and product complexes of several PDEs including PDE4B, PDE4D and PDE5A.

3.2 Active site anatomy

The active site pocket of PDE4 is ~ 15 Å deep and has an opening of ~ 20 Å by 10 Å [102]. The surface area and volume of this pocket are ~ 670 $Å^2$ and 440 $Å^3$, respectively. The active site contains 12 out of 17 absolutely conserved residues across all 21 PDE gene family members [97]. There are three clusters of residues in the active site that are responsible for three distinct functions of PDEs (**Figure 7c**) [100]: (1) nucleotide recognition: a cluster of residues that controls the orientation of the amide group of an invariant glutamine for selective binding to either cAMP or cGMP; (2) substrate binding: two highly conserved hydrophobic residues form a hydrophobic clamp that sandwich the planar purine ring in the substrate (**Figure 7d**); (3) hydrolysis: a cluster of absolutely conserved residues near the di-metal centre that are responsible for cyclic nucleotide hydrolysis.

The active site can be subdivided into three pockets [102] (**Figure 7b**): a metal-binding pocket (M-pocket); a solvent-filled

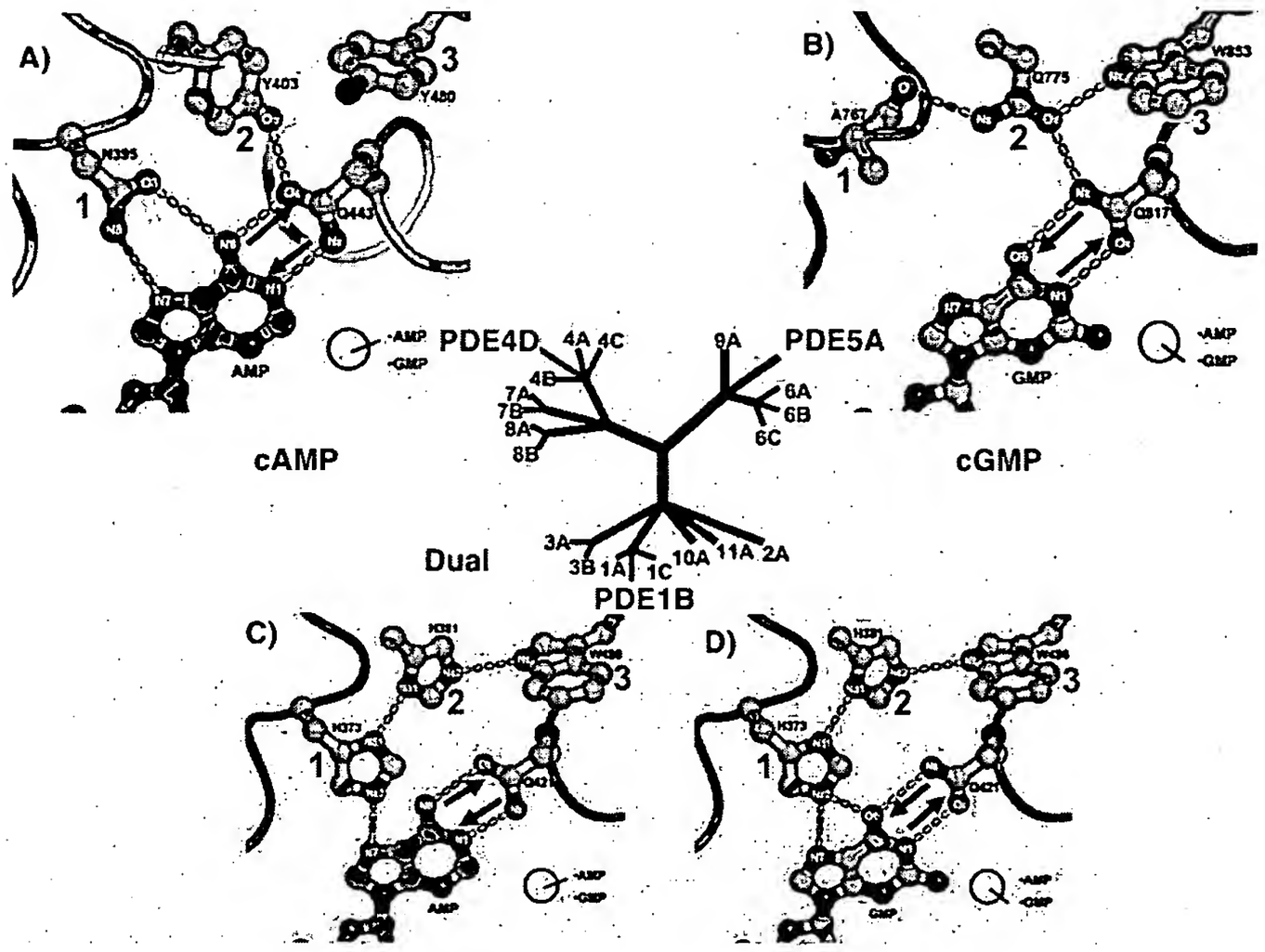

Figure 8. A glutamine switch mechanism of nucleotide selectivity. The three residues that control the orientation of the invariant glutamine and confer nucleotide selectivity are labelled as 1, 2 or 3 in figures 8 and 9. **A**) Q443 recognising AMP in PDE4B by forming a bidentate H-bond with adenine. Specifically, the Nε atom of Q443 donates an H-bond to the N1 atom of the adenine ring and the Oε accepts a H-bond from N6 in the exocyclic amino group of adenine. This particular orientation of Q443 is stabilised by H-bonding of Oε to the phenolic hydroxyl Oη of Y403. In addition, N395 forms a bidentate H-bond with the adenine base by donating one H-bond from Nδ to N7 of the adenine base and accepting one H-bond from the N6 of the exocyclic amino group to its Oδ. **B**) Q817 recognising GMP in PDE5A by forming a bidentate H-bond with guanine. The particular orientation of the Q817 side chain is anchored by its H-bond interaction with Q775. The orientation of Q775 side chain is in turn anchored by the H-bond between Nε in Q775 and the carbonyl oxygen in A767 and the H-bond between Oε of Q775 and the Nε of W853. **C**) Q421 recognising AMP in the model of AMP bound to PDE1B. **D**) Q421 recognising GMP in the model of GMP bound to PDE1B. In (**C**) and (**D**), there are no supporting residues to anchor the orientation of the key glutamine residue thus conferring PDE1B dual-specificity.
AMP: Adenosine monophosphate; GMP: Guanosine monophosphate; PDE: Phosphodiesterase.
Adapted from ZHANG KYJ *et al.*: A glutamine switch mechanism for nucleotide selectivity by phosphodiesterases. *Mol. Cell* (2004) **15**:279-286 [100].

side pocket (S-pocket); and a pocket containing the purine-selective glutamine and hydrophobic clamp (Q-pocket). The M-pocket contains the di-metal ions and highly conserved hydrophobic and polar residues that coordinate the metal ions. The S-pocket consists mainly of hydrophilic amino acids and is filled with a network of water molecules in most of the inhibitor complexes. The Q-pocket can be further divided into three distinct areas: a narrow passage (Q_P) formed by the P-clamp that only a planar structure would fit in and reach through to interact with the conserved nucleotide-selective glutamine, flanked by two asymmetrical hydrophobic subpockets (Q_1 and Q_2).

3.3 Nucleotide selectivity

PDEs achieve their nucleotide selectivity through a 'glutamine switch' mechanism [100]. The amide group of an invariant glutamine adopts one orientation to interact with cAMP and switches to another orientation by flipping 180° to interact with cGMP. This 'glutamine switch' is controlled by an intricate network of hydrogen bonds (H-bond) near this absolutely conserved glutamine.

In the cAMP-selective PDEs, such as PDE4B, the absolutely conserved Q443 forms a bidentate H-bond with the adenine moiety (Figure 8a). This orientation of Q443 is stabilised by H-bonding of Oε to the phenolic hydroxyl group Oη of Y403. The adenine ring also forms a bidentate H-bond with N395 in PDE4B further stabilising the nucleotide binding.

By contrast, in the cGMP selective PDEs, such as PDE5A, the orientation of the key glutamine Q817 is switched from that in PDE4B to allow H-bonding specific to the guanine ring (Figure 8b). This orientation of Q817 is constrained by its H-bonding with Q775. The orientation of Q775 is, in turn, determined by the two H-bonds that it forms with A767 and W853. This intricate network of H-bonds determines the orientation of the γ-amide group of Q817 that is favourable for cGMP, but unfavourable for cAMP binding in PDE5A.

The dual-specificity of some PDEs such as PDE1B is due to the rotational freedom of the amide group of the invariant glutamine. The key glutamine, in this case Q421, can adopt either of the orientations observed in the PDE4 and PDE5 structures, because there is no H-bond network to

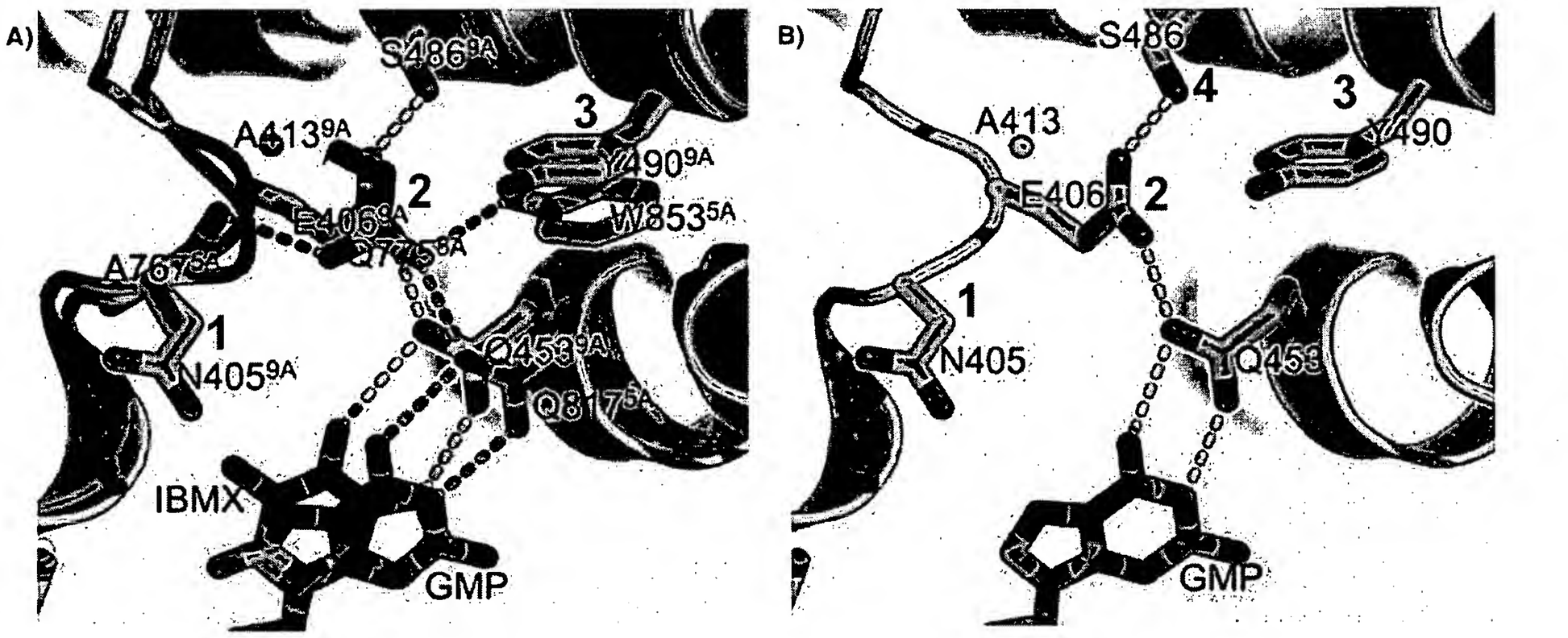

Figure 9. Nucleotide recognition by PDE9A. A) Superposition of PDE9A + IBMX (yellow) and PDE5A + GMP (green) co-crystal structures. Although Q775^{5A} corresponds to A413^{9A} according to sequence alignment, the side chain of Q775^{5A} is occupied by that of E406^{9A}. **B)** The nucleotide recognition by PDE9A is achieved through the same glutamine switch mechanism where the orientation of Q453^{9A} is anchored by forming H-bond with E406^{9A} which, in turn, is H-bonded to S486^{9A} in position 4. The side chain of N405^{9A} has swung away because it could not form bidentate H-bonds with the guanine base.
GMP: Guanosine monophosphate; IBMX: 3-Isobutyl-1-methylxanthine; PDE: Phosphodiesterase.

constrain the orientation of its γ-amide group (Figure 8c and 8d). The three residues that control the orientation of the invariant glutamine and confer nucleotide selectivity in PDE4B, PDE5A and PDE1B are referred to as position 1, position 2 or position 3 and labelled as 1, 2 or 3, respectively, in Figures 8 and 9.

This 'glutamine switch' mechanism can account for the cyclic nucleotide selectivity for all the PDEs [100]. The key residues involved in cyclic nucleotide recognition identified through sequence alignment have revealed a pattern that fit the 'glutamine switch' mechanism. All the cAMP-selective PDEs have an asparagine residues at position 1 (corresponding to N395 in PDE4B in Figure 8a) that can form a bidentate H-bond with adenine, but not with guanine. All the cGMP-selective PDEs, with the exception of PDE9A, have a glutamine at position 2 (corresponding to Q775 in PDE5A in Figure 8b). This glutamine anchors the orientation of the invariant glutamine (Q817 in PDE5A in Figure 8b) to form bidentate H-bond to guanine, but not with adenine. All the dual-specific PDEs have neither asparagine at position 1 nor glutamine at position 2 to confer cyclic nucleotide selectivity. PDE9A seems to represent an exception to the rule above, because the presence of N405 at position 1 and A413 at position 2 would suggest that PDE9A should be cAMP-selective rather than cGMP-selective. This mystery of cGMP selectivity for PDE9A has been unveiled when the co-crystal structure of PDE9A bound to IBMX was reported [103]. PDE9A uses the same 'glutamine switch' mechanism as that for PDE5A to gain selectivity for cGMP except the key residues that anchor the orientation of the invariant glutamine are different from what were inferred from sequence alignment (Figure 9a). The mutation of Q775 in PDE5A to A413 in PDE9A has created a void in the structure that was filled by the side chain of E406 in position 2 which forms H-bonds with S486 in position 4 and the amide nitrogen of Q453 (Figure 9a). Therefore, E406 serves the role of anchoring the orientation of the invariant glutamine Q453 that is favourable for cGMP, but not for cAMP (Figure 9b). The structure also revealed that N405 in position 1, which would be favourable for cAMP over cGMP, has swung away and is not within H-bond distance to the nucleotide (Figure 9b).

3.4 Mechanism of hydrolysis

The mechanism of cyclic nucleotide hydrolysis has been proposed to proceed with a nucleophilic attack of the cyclic phosphate by a hydroxyl which is derived from an activated water molecule. Computational calculations based on the first structure of the apo catalytic domain of PDE4B has revealed that the bridging water between Zn^{2+} and Mg^{2+} ions is a hydroxyl which is the attacking nucleophile in the hydrolysis [104]. There are still many questions remaining in the hydrolysis mechanism. Does the reaction proceed in an SN1-like (dissociative) or SN2-like (associative) mechanism? Which are the proton donor and acceptor? Where does the proton go and where does it come from? How is the intermediate state stabilised?

Recent co-crystal structures of PDE4B and PDE4D in complex with the catalytic product AMP have shed some light on the hydrolysis mechanism which involves the bridging hydroxyl as the attacking nucleophile [98,99] (Figure 10a). An invariant aspartate (D392^{4B}) serves as a general base to

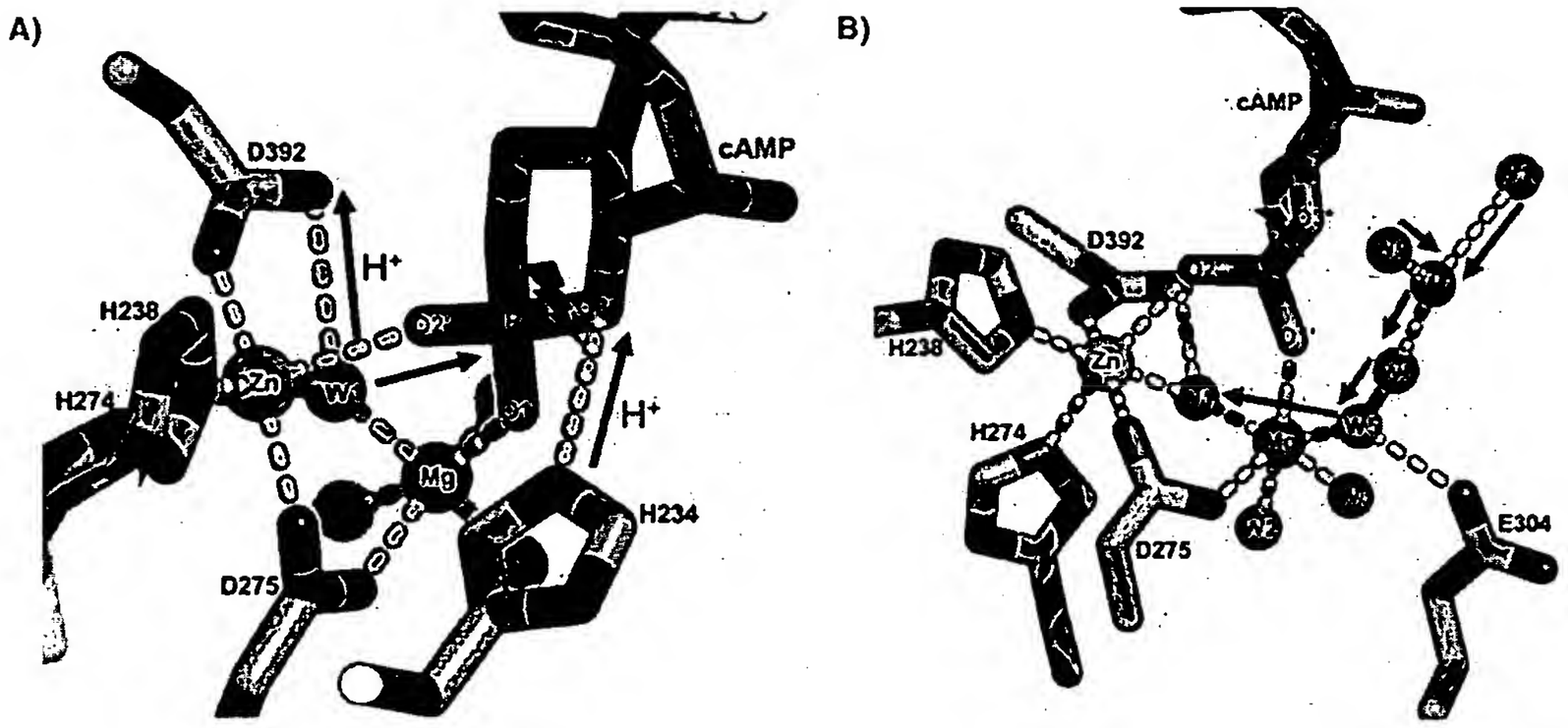

Figure 10. Mechanism of hydrolysis for PDE. A) The mechanism of cyclic nucleotide hydrolysis is illustrated on the modelled cAMP bound to PDE4B. D392 deprotonates the bridging water W1 to generate a nucleophile that attacks the phosphorous P inline to the scissile bond. The leaving O3' is protonated by H234. **B)** The Mg^{2+} coordinated water molecule, W5, forms a H-bonded water network leading to the solvent. This W5 could replenish the bridging hydroxyl, W1, at the end of each catalytic cycle.
cAMP: Cyclic adenosine monophosphate; PDE: Phosphodiesterase.

deprotonate the bridging water activated by the coordinated Zn^{2+} and Mg^{2+} ions, the resulting hydroxide may act as the nucleophile that attacks phosphorus through an inline associative mechanism. The formation of a penta-coordinated intermediate or transition state could be stabilised by the interaction of two oxygen atoms on the phosphorus with the metal ions. The O3' leaving group is protonated by the general acid $H234^{4B}$, which is found to be hydrogen bonded to O3' in many of the co-crystal structures of PDE4B and PDE4D in complex with AMP, as well as PDE5A in complex with GMP [98-100]. Not only $H234^{4B}$ is absolutely conserved in all PDEs, but the three residues that it interacts with are also functionally conserved. The PDEs have committed four amino acids to donate one proton, which highlights the importance of this protonation step in the evolution of these enzymes to catalyse cyclic nucleotide hydrolysis. Xu *et al.* [99] noted that the hydrolysis may also proceed by a dissociative mechanism, leading to the formation of a metaphosphate-like intermediate, where breaking the scissile bond would precede the nucleophilic attack by the hydroxyl on the phosphorus. This intermediate would be stabilised by the metal ions. The associative and dissociative mechanisms may represent two extremes; in reality, the transition state may lie between associative and dissociative-like.

An alternative nucleophile was proposed to be a water molecule (W5) bound to the Mg^{2+} ion [98]. In this mechanism, $E304^{4B}$ acts as a general base to activate this water for nucleophilic attack on the phosphorus atom, whereas $H234^{4B}$ serves as a general acid to donate a proton to the O3' oxygen. The mutation of $E304^{4B}$ has been shown to significantly reduce the catalytic activity of PDE4B [105]. However, it is also possible that the role of $E304^{4B}$ in hydrolysis is a gatekeeper that regulates the supply of water, the Mg^{2+} bound water molecule W5, to replenish the bridging hydroxyl at the end of each catalytic cycle (Figure 10b).

The elucidation of the precise mechanism of cyclic nucleotide hydrolysis would benefit from the knowledge of co-crystal structures of the catalytic domain of PDEs in complex with the substrate, cAMP and cGMP, as well as their transition state analogues.

3.5 Structural basis of inhibitor potency and selectivity

There has been a flurry of co-crystal structures of PDE4 bound to various known inhibitors reported in the literature recently [99,100,102,106-108]. These co-crystal structures have enriched our understanding of the driving force behind inhibitor binding and have enabled the structure-based design of more potent and selective PDE4 inhibitors. There are two common features of inhibitor binding to PDEs [102]: 1) a planar ring structure of the inhibitor that is held tightly in the active site by a pair of hydrophobic residues that form a hydrophobic clamp, and 2) hydrogen bond interactions with an invariant glutamine residue that is essential for nucleotide selectivity [100]. These two common features define the scaffold of all known PDE inhibitors. The ability to identify the scaffold through the formation of conserved interactions with the protein target has led to the scaffold-based discovery of new PDE4 inhibitors [109].

All the reported inhibitors bound to PDE4 represent four scaffold classes: catechol diether, xanthine, pyrazole and purine analogues. The majority of these co-crystal structures are dialkoxyphenyl derivatives [99,102,106,108] (Figure 11) and only one is a xanthine derivative, IBMX [107] (11, Figure 11h). The superposition of the co-crystal structures of the dialkoxyphenyl

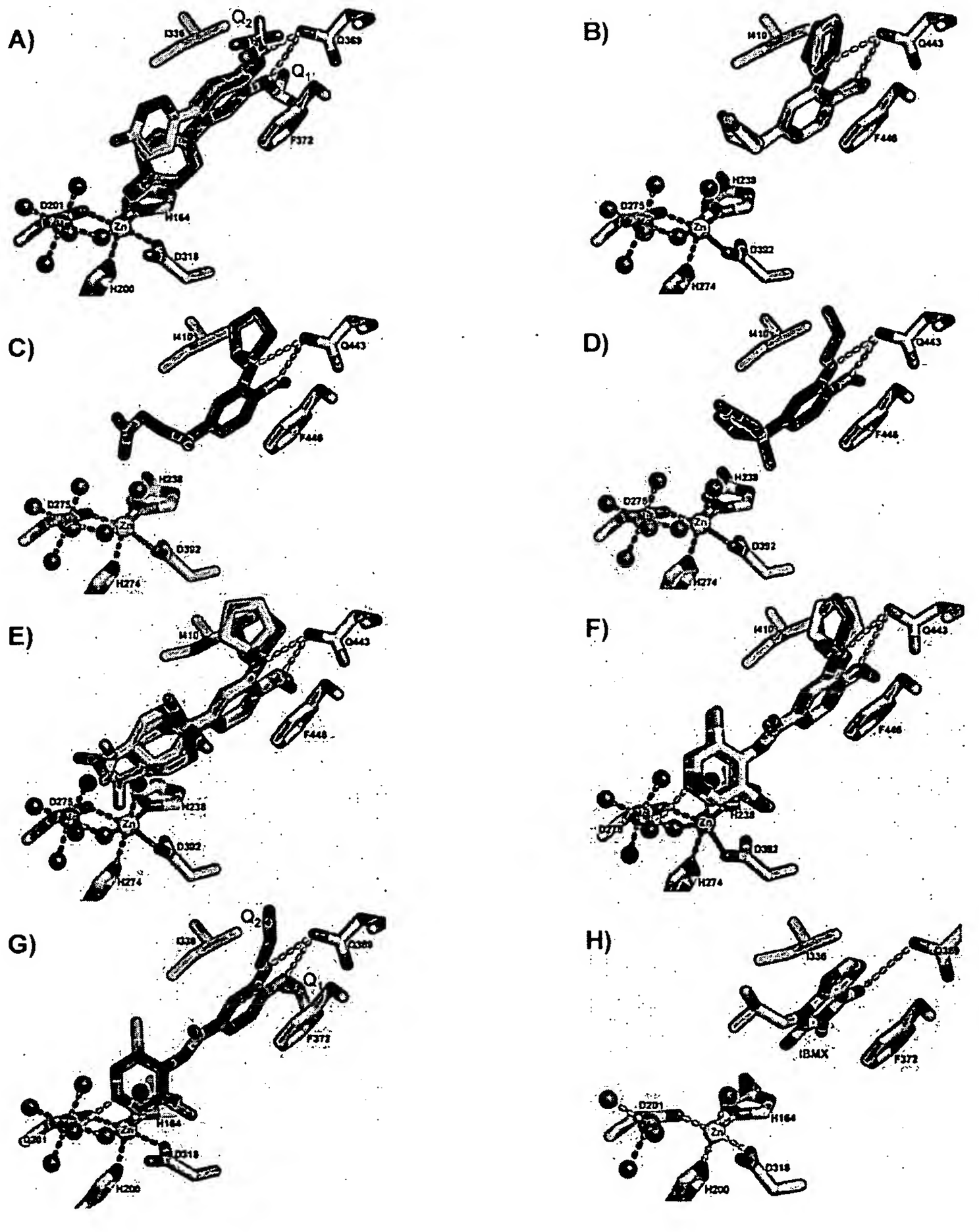

Figure 11. Co-crystal structures of PDE4 inhibitors. A) Structure of zardaverine bound to PDE4D. Three conformations of zardaverine have been observed and their carbon atoms are coloured in green, cyan and yellow, respectively. Predominant conformations are represented by green and cyan that have their difluoromethoxy group pointing up to the Q2-pocket and their pyridazinone group pointing to the di-metal ions and coordinated to Zn^{2+} by displacing the bound water molecule. The minor conformation is represented by yellow which has its difluoromethoxy group pointing down to the Q1 pocket and its pyridazinone group pointing away from the di-metal ions. **B)** Structure of rolipram bound to PDE4B: co-crystal structures with (R,S)-rolipram (green) or (R)-rolipram (yellow). Only one binding mode – with the pyrrolidinone group pointing away from the di-metal ions – has been observed. **C)** Structure of filaminast bound to PDE4B. The hydroxamate substituent extends to the M-pocket and tilted slightly up towards the opening of the pocket. **D)** Structure of mesopram bound to PDE4B. The cyclic carbamate substituent extends to the M-pocket and tilted slightly up towards the opening of the pocket. **E)** Structure of cilomilast bound to PDE4B and PDE4D. The carboxocyclohexyl group adopts two conformations in the PDE4D complex. The carbon atoms of cilomilast in PDE4B are in green, and those of cilomilast in PDE4D are in yellow and cyan. **F)** Structure of roflumilast bound to PDE4B and PDE4D. The carbon atoms of roflumilast in PDE4B are in green and the carbons of roflumilast in PDE4D are in yellow. The difluromethoxy group binds at the Q1 pocket, whereas the cyclopropyl methyl group binds at the Q2 pocket. The dichloropyridyl group extends to the di-metal ion site and forms one H-bond to a water molecule that is coordinated to Mg^{2+}. **G)** Structure of piclamilast bound to PDE4B and PDE4D. The carbon atoms of piclamilast in PDE4B are in green and the carbons of piclamilast in PDE4D are in yellow. **H)** IBMX bound to PDE4D. IBMX is sandwiched by the hydrophobic clamp formed by I336 and F372 and forms a hydrogen bond with Q369.

IBMX: 3-Isobutyl-1-methylxanthine; PDE: Phosphodiesterase.

family of compounds, including zardaverine (19, Figure 11a), rolipram (1, Figure 11b), filaminast (25, Figure 11c), mesopram (18, Figure 11d), cilomilast (3, Figure 11e), roflumilast (4, Figure 11f) and piclamilast (20, Figure 11g), with PDE4B and PDE4D revealed that the scaffold is a catechol diether that forms a H-bond with the purine-selective glutamine and is also sandwiched by the P-clamp. The catechol diether scaffold superposed extremely well in all of these co-crystal structures, whereas the substituents showed significant variations in their binding conformation, as well as in the residues that they interact with. The various substituents on the catechol diether scaffold explore the deep pocket close to the metal-binding site, and how well they form interactions with residues lining this pocket determines their relative binding affinity. The relatively smaller pyrrolidinone substituent in rolipram has resulted in a relatively lower binding affinity (570 nM in the case of PDE4B). The more potent dialkoxyphenyl compounds have larger substituents that could form more favourable interactions with residues lining the relatively large M-pocket. The most potent compounds, such as roflumilast and piclamilast, reach deep into the M-pocket, not only interacting with residues near the metal ion, but also forming H-bond with a water molecule coordinated to the metal ion. A surprise finding from these co-crystal structures is that all the inhibitors, with the only exception of zardaverine, do not directly interact with the metal ions contrary to what was expected by many people in the field.

The analysis of these co-crystal structures of inhibitor and PDE4 complexes have shown that the interactions with residues lining the two hydrophobic subpockets near the invariant purine-selective glutamine are important for inhibitor binding. The inhibitor potency can be further increased by exploring interactions with residues near the di-metal ion centre, as well as through the formation of water-mediated interactions with the metal ions [102].

The design of inhibitors selective for PDE4B over PDE4D remains a very challenging task. All the inhibitors co-crystallised with PDE4 catalytic domains are nonselective between PDE4B and PDE4D. These nonselective inhibitors bound to PDE4B and PDE4D in nearly identical mode, confirming their nonselectivity. Perhaps the co-crystal structures of those PDE4D selective inhibitors, such as NVP-ABE171 [60] and CP-671305 [61], will shed some light on their PDE4D selectivity and point to the way of designing PDE4B-selective inhibitors. The residues in the active site of PDE4B and PDE4D that are in contact with various inhibitors are identical. Moreover, the conformational differences in the active site between various PDE4B and PDE4D structures are very subtle. It would appear that there are several possible ways of designing inhibitors with PDE4B/PDE4D selectivity. The first is to exploit the subtle differences in the conformational state of residues in the active site of PDE4B and PDE4D and the differences in their potential of induced fit in response to inhibitor binding. The second is to design inhibitors that make contact with residues beyond these from the catalytic domain, such as the C-terminal domain or the N-terminal regulatory domains which may fold near the active site. The third is to explore potential conformational changes in the active site of the catalytic domain triggered by the presence of the N-terminal regulatory domains in the full-length enzyme.

Most of the reported PDE4 inhibitors are nonselective against PDE7 (PDE7A, PDE7B) due to the high degree of sequence homology (85 – 95% sequence identity) between these two subfamilies of enzymes. In fact, many of the documented PDE7 inhibitors are either PDE4 inhibitors or close analogues thereof. Although the crystal structure of the catalytic domain of PDE7 is not available, homology modelling of PDE7 based on the structure of PDE4 has revealed several residue differences in the active site that could be exploited for the design of selectivity between PDE4 and PDE7. The discovery of PDE7-selective inhibitors, BRL-50481 and BMS-586353, attests to this possibility [110]. Although the association of PDE7A with IL-2 production and the proliferation of anti-CD3- and anti-CD28-stimulated human T lymphocytes suggests the potential of PDE7A as a novel anti-inflammatory drug target [111,112], it was found that PDE7A-deficient mice have functional T cells [113]. Selective PDE7 inhibitor, BRL-50481, was found to have no effect on the proliferation of $CD8^+$-T-lymphocytes, however, acted synergistically with PDE4 inhibitors in the suppression of TNF-α release [110]. Consequently, several dual inhibitors of PDE4 and PDE7 have been developed to explore the possibility of obtaining more efficacious drugs for the treatment of inflammatory diseases [210,211].

3.6 Scaffold-based design of PDE4 inhibitors

The structure-based insight into the inhibitor binding to PDE4 has facilitated the rational design of more potent and selective PDE4 inhibitors. A scaffold-based drug discovery paradigm was applied to the identification of a pyrazole family of PDE4 inhibitors [109]. This scaffold-based drug discovery method starts with low affinity screening of a low molecular weight compound library followed by high-throughput co-crystallography of the screening hits to select compounds that exhibit a dominant binding mode and have appropriate sites for substitution. These compounds serve as scaffolds that are the starting point for lead optimisation.

Starting from a core library of ~ 20,000 low molecular weight compounds, a high-throughput biochemical assay was used to screen against a representative subset of PDE family members. Compounds that showed > 30% inhibition at 200 µM against three or more PDEs in the screening panel were all set up for co-crystallisation with PDE4D and PDE4B. Co-crystal structure of a low-affinity 3,5-dimethyl-1H-pyrazole-4-carboxylic acid ethyl ester (PCEE) with PDE4D has revealed its potential as a scaffold candidate [100,102] (Figure 12a).

This scaffold candidate PCEE was further tested through a validation process by first identifying potential sites of substitution based on the ability to make favourable chemical interactions in the available space. Consequently, a

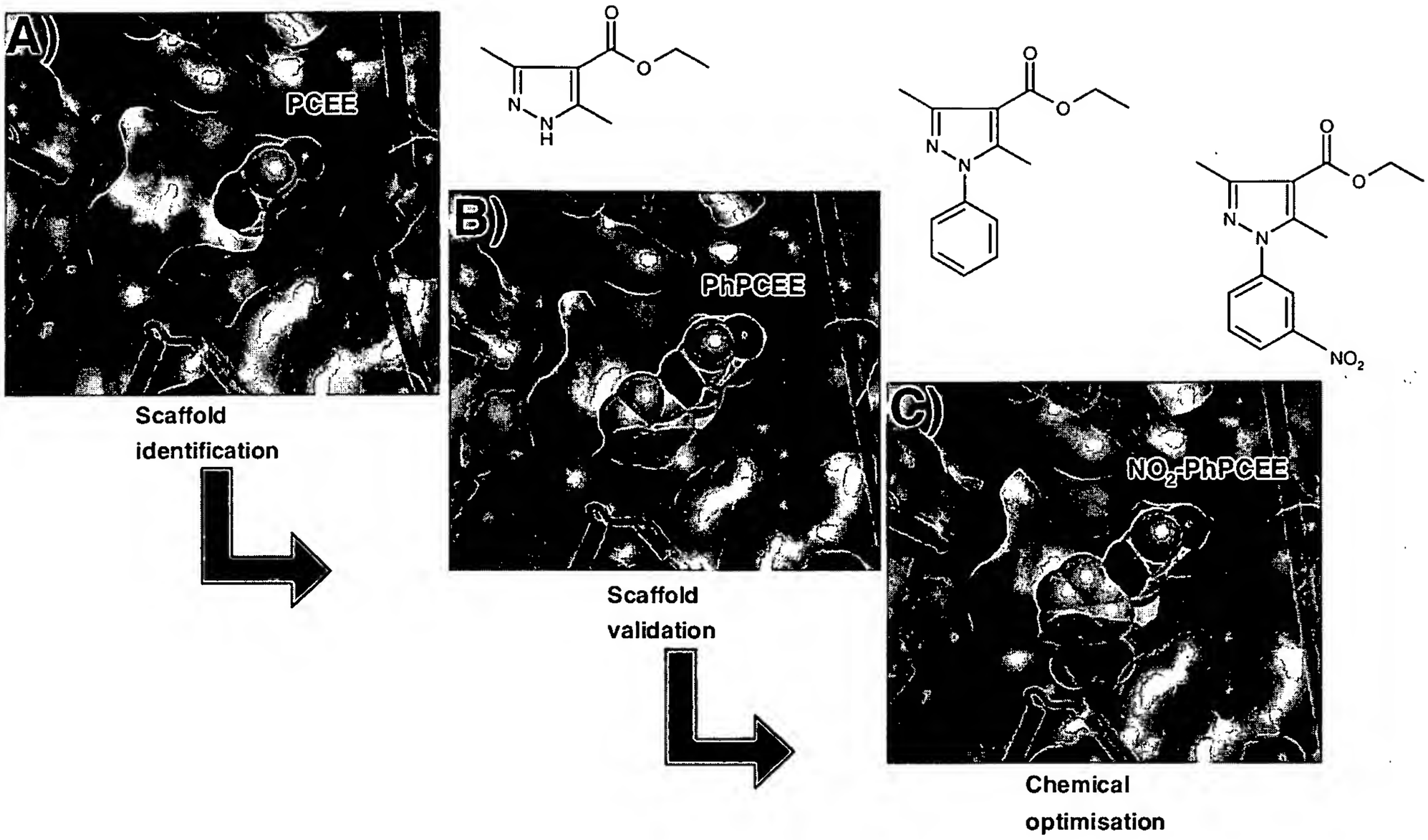

Figure 12. Scaffold-based lead discovery of pyrazoles as PDE4 inhibitors. A) Scaffold identification. The scaffold candidate, 3,5-dimethyl-1H-pyrazole-4-carboxylic acid ethyl ester (PCEE), is a weak PDE4D inhibitor with IC_{50} of 82 μM. **B)** Scaffold validation. The derivative of the scaffold, 3,5-dimethyl-1-phenyl-1H-pyrazole-4-carboxylic acid ethyl ester (PhPCEE) has significantly increased potency towards PDE4D with IC_{50} of 0.27 μM. **C)** Chemical optimisation. The validated scaffold was optimised into a potent PDE4D inhibitor, 3,5-dimethyl-1-(3-nitro-phenyl)-1H-pyrazole-4-carboxylic acid ethyl ester (NO_2-PhPCEE), with IC_{50} of 0.021 μM. Compounds are represented by solid surface coloured by atomic types. The active site pocket is represented by a molecular surface. The PDE4D is represented by cartoons where helices are shown as cylinders and loops are shown as tubes.

IC_{50}: Half maximum inhibitory concentration; PCEE: 3,5-Dimethyl-1H-pyrazole-4-carboxylic acid ethyl ester; PDE: Phosphodiesterase.

Adapted from HOUSLAY MD, SCHAFER P, ZHANG KYJ: Phosphodiesterase-4 as a therapeutic target. *Drug Discov. Today* (2005) **10**:1503-1519 [114], with permission from Elsevier.

small set of compounds with substitutions at the 1-, 3- or 5-positions of the pyrazole were synthesised and tested in the PDE assay. A derivative compound, 3,5-dimethyl-1-phenyl-1H-pyrazole-4-carboxylic acid ethyl ester (PhPCEE) was selected for further optimisation of potency. The co-crystal structure of this derivative compound PhPCEE in complex with PDE4D has validated the PCEE moiety as a scaffold by demonstrating that the phenyl substitution does not change the binding mode of the pyrazole to PDE4 (Figure 12b).

More than 100 compounds were computationally designed and docked into the active site pocket, based on the co-crystal structure of PhPCEE with PDE4D. After eliminating a large number of substitutions that were predicted to cause undesirable interactions with residues in the active site, a total of 10 compounds were synthesised with a predicted increase in binding affinity due to the formation of favourable interactions. The co-crystal structures of 4 of the 10 synthesised molecules (Figure 12c) have been determined. These co-crystal structures of PhPCEE derivatives have proven that the computational design of compounds and the prediction of structure–activity relationship to be accurate.

Using a scaffold as a starting point, potent 2-Cl- and 3-NO_2- PhPCEE (IC_{50} 20 nM in PDE4D) have been obtained in two rounds of chemical synthesis and only a total of 21 compounds had to be derived, starting from the initial PCEE scaffold (IC_{50} 82 μM in PDE4D). The total molecular weight added from the initial scaffold candidate to the final compound is only 121 Da, which has resulted in a 4000-fold potency increase. The scaffold-based lead discovery method has the potential to be used widely to expedite the lead discovery effort for many other targets for which known small-molecule modulators are limited.

4. Expert opinion

As a key regulator of intracellular signalling, PDE4 has become a validated target for the development of therapeutics for inflammatory diseases such as asthma and COPD. PDE4

has also been shown to be a potential therapeutic target for depression, memory enhancement, cardiovascular disease and osteogenesis. The development of PDE4 therapeutics has evolved from first-generation weak and nonselective inhibitors to late-generation highly potent and selective inhibitors. The recent advances in the clinical development of cilomilast and roflumilast attest to the achievement of these efforts. However, the side effects of emesis and nausea have limited the therapeutic window of these inhibitors. The differential tissue expression and subcellular localisation of PDE4 isoforms, along with genetic knockout experiments, have implicated isoform-selective inhibitors as a possible way to achieve increased therapeutic index by reducing the side effect of emesis and nausea. The difficulty of discovering PDE4B-selective inhibitors has, so far, prevented the testing of this hypothesis. Therefore, achieving PDE4B isoform selectivity could be the focal point in the development of the next generation of PDE4 inhibitors. It should be noted that other means of achieving increased therapeutic window include reduced penetration of the blood–brain barrier, and reduced binding affinity to the HARBS form of PDE4. Although emesis and nausea have been the major side effects in PDE4 inhibitor development, recent data have pointed out that cardiac abnormalities and vasculitis associated with the long-term administration of PDE4 inhibitors could pose even more serious challenges. Recent data, including the link of PDE4D polymorphism to stroke, the involvement of PDE4 in vascular smooth muscle proliferation, and the link between PDE4 and osteogenesis, have opened new windows of opportunity for the discovery of PDE4 inhibitors as potential therapeutics for the treatment of cardiovascular diseases and osteoporosis. However, further experiments are needed to confirm these linkages. There is still a long road ahead for the validation of PDE4 as a therapeutic target for these diseases which requires the development of inhibitors that have clinically proven efficacy.

Recent advances in the structural understanding of the PDE4 catalytic domain and its inhibitor binding properties will have a profound impact on the design of next generation PDE4 inhibitors. The design of PDE4 isoform selective inhibitors, which is the new frontier in PDE4 inhibitor development, will greatly benefit from this structural information. The catalytic domain structures of PDE4B and PDE4D have revealed a high degree of similarity in their active sites although there are subtle differences that could be exploited for selectivity. It is conceivable that the active site might be reshaped or remodelled due to the impact of the regulatory domains where more significant differences exist among isoforms. The crystal structure of the full-length PDE4 isoforms will shed light to this and potentially provide further insight to the design of isoform selective inhibitors. We have just begun to witness the impact of structural information on the design of PDE4 inhibitors and we expect more inhibitors will be designed based on these structural insights in the future.

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